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THE SOURCE OF PHOSPHORUS IN BACTERIOPHAGE*

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Until recent years, very little was known concerning virus metabolism or the source of materials used by viruses for metabolic purposes, and physical and chemical methods were used mainly for the characterization of virus particles. These methods have contributed a great deal to our knowledge of viruses. However, some later investigations on influenza virus, mouse pneumonitis virus, and Rous sarcoma virus have shown that purified preparations of these agents may contain antigens and chemical substances of the normal host tissue, bound, perhaps chemically, to other substances characteristic of the virus alone.[2, 9, 14, 19, 20] These results suggest a need for re-interpretation of earlier chemical analyses of the various viruses.

Recently, certain investigators have turned their attention to the study of the metabolism of viruses. Thus, for example, studies of the enzyme content of various virus preparations have shown that the viruses themselves probably are incapable of carrying out the necessary metabolic reactions for production and utilization of energy. This leads to the conclusion that the host cells must furnish such enzymes.[28]

Price,[21, 22, 24] working with *Staphylococcus muscae* and a bacteriophage to which this strain was susceptible, has attempted to demonstrate growth requirements of bacteriophage through additions of specific medium components to penicillin-inhibited bacteria. Specific enzyme poisons have also been used by some investigators, such as Price,[23] Fitzgerald and Lee,[12] and Fitzgerald and Babbit,[11] in attempts to clarify problems of bacteriophage metabolism. Cohen and Fowler[7] have concluded as a result of their investigations that the synthesis of tryptophane is necessary for the growth of T_2 bacteriophage. They further concluded that tryptophane need not be present as a component of the host's external environment but must be present in the environment to which the virus is exposed. These investigators have

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also shown[8, 13] that addition of other amino acids as well as purines and pyrimidines to a minimal medium stimulates the production of virus and that the rate of synthesis of these compounds from simpler ones in the minimal medium determines the rate and extent of growth of the virus.

In investigations on the phosphorus metabolism of bacterial viruses, Cohen, [3, 4, 5] using P^{32} as a tracer element, found that the main source of phosphorus used in the formation of T_2r^+ and T_4r^+ bacteriophage was to be found in components of the medium in which the bacteria and viruses were suspended rather than in compounds synthesized by the bacteria prior to their infection with virus. However, a certain percentage of the phosphorus found in the bacteriophage seems to have been derived from the latter source. These results were confirmed by Putnam and Kozloff,[25] who found that approximately 23% of the phosphorus of virus T_6r^+ was derived from bacterial phosphorus, and the rest was derived from the medium.

Cohen,[3, 4, 5] as well as Putnam and Kozloff,[25] purified their virus preparations mainly by means of differential centrifugation followed by dialysis. The specific activity of the total virus phosphorus as well as that of the desoxyribonucleic acid phosphorus was then determined and compared with specific activities of the host cells and of the media.

In view of the fact that only lysis inhibiting strains of bacterial virus were used by the abovementioned investigators, it was felt that similar investigations on a non-lysis inhibiting strain of bacteriophage might furnish valuable additional information as to some of the host-parasite metabolic relationships. It was with this purpose in mind that the present investigation was undertaken.

It was also felt that differential centrifugation and dialysis alone might not be sufficient to eliminate contaminating phosphorus-containing compounds from bacteriophage preparations and that these might introduce a considerable source of error into the determination of the phosphorus content of the bacteriophage particles themselves. Therefore, a biological method was used, in combination with the usual physical methods of detection of radioactive tracer elements, which eliminated, to a considerable degree, this source of error. The biological method involved the adsorption of bacteriophage to phage-sensitive cells. These cells were subsequently washed, and the radioactivity of the adsorbed material was measured with a Geiger-Mueller counting apparatus.

These experiments are presented in two sections. In Section I are control experiments, the main purpose of which was to show that the presence of

bacteriophage does not influence bacterial adsorption of phosphorus compounds which are not associated with the bacteriophage particles themselves. In Section II are presented the experiments dealing with the sources from which bacteriophage obtains phosphorus.

Materials and methods

Radioactive phosphorus in the form of KH_2PO_4 or H_3PO_4 was used in the experiments involving adsorption of inorganic phosphates. In other experiments, the phosphorus compounds were derived from bacteria lysed by bacteriophage or by mechanical means.

The medium used for the growth of bacteria and bacteriophage was beef heart infusion broth.

The bacteria used in the adsorption tests were: (1) a strain of *Shigella paradysenteriae* (Flexner 2), henceforth denoted as D43; (2) a variant of D43, denoted as D/Pα, which was resistant to the strain of bacteriophage used in this work; and (3) a strain of *Bacillus mesentericus*.

The bacteriophage, active against D43, was isolated from raw sewage obtained from a sewage disposal plant. This phage will henceforth be denoted as Pα.

The bacteria used in the adsorption tests were grown in bottles, each containing 50 ml. of infusion broth. The cultures were allowed to grow for twenty-four hours at 37° C. and were then sedimented by centrifugation. The packed cells were taken up in physiological saline in such volumes that the number of bacteria per ml. of the different strains of bacteria to be used in any given test was approximately equal, as measured by the turbidity of the suspensions. The turbidities were measured in a Klett-Summerson photoelectric colorimeter.

In some experiments where suspensions of living bacteria were used in the adsorption tests, these suspensions were kept cold (in ice water) in order to prevent growth of either bacteria or bacteriophage. In other experiments the bacterial suspensions were heated prior to their use in adsorption tests in order to attain the same result. Each method gave essentially the same results in subsequent adsorption tests, both as to adsorption of phosphates and of bacteriophage.

For the assay of radioactivity, a Geiger-Mueller counting tube and scaling circuit were used, the tubes being of the bell jar or the cylinder type.* Discs of filter paper, slightly smaller in diameter than the windows of the Geiger-Mueller tube, were placed on glass slides. One-tenth ml. of the material to be assayed, generally of a fluid nature, was spread as uniformly as possible on these discs, which were then allowed to dry. The filter paper discs were finally covered with scotch tape and the slides assayed. Each slide was assayed for a period of at least three minutes on a convenient scale. When the recorder indicated counts of less than double the background, assays were carried out for at least six minutes. For convenience in the comparison of results, all counts were corrected to the scale of 2 by multiplying by the proper factor.

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* This apparatus was constructed at the Department of Physics of Yale University and made available through the courtesy of Dr. Ernest C. Pollard.

Previous to, as well as following, the assay of a sample, the background was determined, and a radioactive uranium glass standard was assayed. Since counts of the uranium glass standard were found to vary considerably from time to time, corrections were made for an arbitrary count of the standard of 360 cts./min. Corrections were also made for radioactive decay whenever experiments using the same source of radioactive phosphorus were performed but at various intervals of time. In addition, all counts as given in the tables were corrected for the background.

Assays were performed on all solutions containing radioactive phosphorus at the beginning of the experiments and at all intermediate steps.

Bacteriophage titrations were performed in the usual manner on solid media, and titers are expressed as the number of plaques times the dilution of the bacteriophage suspension, of which one ml. gave the corresponding number of plaques.

Section I

Studies on the Adsorption by Bacteria of Phosphorus Compounds of Various Types in the Presence and Absence of Bacteriophage

Procedures

Experiment 1

To each of four tubes were added 0.5 ml. of radioactive KH_2PO_4 solution. To two of the tubes were added 4.5 ml. of infusion broth, and to the remaining two tubes were added 4.5 ml. of stock Pα grown in infusion broth. To one tube containing phage, and to one tube containing broth but no phage, were added 2.5 ml. of D/Pα suspension containing about 5×10^{10} cells/ml.,

Table 1

Material assayed	*Ct./min.*
Medium	18,100
Adsorbing suspension:	
D43	146 ± 7.0
D43 + phage	138 ± 6.8
D/Pα	167 ± 7.5
P/Pα + phage	146 ± 7.0
Phage titer before adsorptions	7.0×10^9
Phage titer after adsorption with D43	6.8×10^8
Phage titer after adsorption with D/Pα	6.8×10^9

± values refer to standard deviation.

prepared as described above. To the other two tubes were added like amounts of D43 suspension. After allowing one-half hour for adsorption of phage and phosphates, the cells were thrown down by centrifugation and the sedimented cells washed six times with saline. Following the last washing, the packed cells were transferred to slides for the assay of radioactivity.

The results of this experiment are summarized in Table 1. The presence of bacteriophage did not influence the adsorption of phospate, and the two strains of bacteria did not differ in ability to adsorb the inorganic phosphates.

Experiment 2

To 15 ml. of infusion broth was added 1 ml. of radioactive KH_2PO_4 solution. The resulting mixture was then serially diluted 1/20, 1/80, 1/320, 1/1280, and 1/2560, each dilution being prepared in 10 ml. of broth. Each of the dilutions was divided into two equal parts of 5 ml. each. One portion of each dilution was added to a tube containing packed D43 cells which were free of phage (approximately 1×10^{11} cells per tube), and the other portion was added to a tube containing a like amount of packed D43 cells which had previously been exposed to Pa phage (approximately one particle of bacteriophage per cell). The cells in the tubes were suspended in the radioactive media by vigorous shaking and then allowed to stand one-half hour to allow adsorption of radioactivity. The suspensions were then centrifuged and the bacteria washed six times in saline. Following the last washing, the packed cells were transferred to slides for assay of radioactivity.

TABLE 2

Dilutions of radioactive medium	*Ct./min.*	*Packed cells Ct./min.*	*Packed cells + phage Ct./min.*
Undiluted	268,160	1,400	1,432
1/20	13,408	82	68
1/80	3,352	14	16
1/320	584	4	4
1/640	300	1	2
1/1280	154	2	2
1/2560	72	1	3
1/5120	31	3	1

Phage titer before adsorptions: 8×10^8
Phage titer after adsorptions: $7 \times 10^{8*}$

* Average values of all the tubes. Variation of titers was within the range of experimental error.

The results of this experiment are summarized in Table 2. As in Experiment 1, the presence of bacteriophage did not influence the adsorption of phosphate, nor did the two strains of bacteria differ in their ability to adsorb the inorganic phosphates.

Experiment 3

One-tenth ml. of a solution of radioactive KH_2PO_4 was added to 10 ml. of infusion broth. The medium was then inoculated with D43 and incubated at 37° C. for eighteen hours. Following incubation, the culture was centrifuged at 3000 r.p.m. for ten minutes, the supernatant decanted, and the sedimented cells washed six times with saline. The final sediment was suspended in 20 ml. of infusion broth with the addition of 0.1 ml. of stock P_α phage, and the suspension was placed in the 37° incubator. After one and one-half hours of incubation, lysis was complete. The lysate was clarified by centrifugation and used in adsorption tests.

Dilutions of the solution of radioactive KH_2PO_4 were prepared in infusion broth. These dilutions were 1/10, 1/100, 1/200, 1/400, and 1/800, each in a volume of 20 ml. These solutions were used in the adsorption tests run in parallel with the test using the radioactive lysate of D43 cells prepared through the action of bacteriophage. The amount of cells used in each tube was one-sixth that used in the foregoing experiment, but otherwise the procedures of the experiments were identical.

The results of this experiment are summarized in Table 3 and will be discussed in later pages.

TABLE 3

Dilutions of P^{32} solution	*Ct./min.*	*Cells from corresponding solution after washing Ct./min.*
1/10	120,800	161
1/100	12,080	100
1/200	6,026	57
1/400	3,064	27
1/800	1,570	7
Lysate	77	91

Experiment 4

A radioactive lysate was prepared in a manner identical with that in Experiment 3. Three strains of bacteria were used in this experiment for

the adsorption tests: D43, D/Pa, and a strain of *Bacillus mesentericus* obtained from the stock culture of the Department of Bacteriology of the Yale University School of Medicine. These bacteria were grown in infusion broth and prepared for the adsorption tests as described under Materials and Methods. The number of cells of each strain used in each tube was approximately 1×10^{11}. The adsorption procedures used were as described in earlier experiments.

The results of this test are summarized in Table 4. This table shows that not all the phosphates released from bacterial cells on lysis by bacteriophage are associated with the latter, since cells which did not adsorb the virus took up as much radioactivity as did bacteria which adsorbed 90% of the bacteriophage.

TABLE 4

	Ct./min.		
Lysate	91	Titer of bacteriophage in lysate, before adsorption	1.1×10^{10}
Cells which had been suspended in lysate and then washed:		Titer of lysate after adsorptions with D43	9.8×10^{9}
D43	74 ± 5.4	Titer of lysate after adsorption with D/Pa	9.9×10^{9}
D/Pa	67 ± 5.2		
B. mesentericus	76 ± 5.5	Titer of lysate after adsorption with *B. mesentericus*	1.0×10^{10}

± values refer to standard deviation.

Experiment 5

Two radioactive bacterial lysates were used in this experiment. One was prepared in a manner identical with that used in Experiments 3 and 4. The other was prepared by sonic disintegration of the cells, as follows: One ml. of a sterile solution of radioactive KH_2PO_4 was pipetted into 20 ml. of infusion broth. This medium was inoculated with D43 and incubated at 37° C. for twenty-four hours. At the end of this time, the cells were thrown down by centrifugation and the sedimented cells washed six times in saline. After the last washing, the cells were re-suspended in 10 ml. of infusion broth and transferred to a lusteroid cup cut down to fit the cup of a Raytheon Magnetostrictor. This apparatus was that described by Shropshire.[27] The lusteroid cup was placed in the Magnetostrictor, surrounded by water, and the current was turned on. The apparatus was run

at its maximum frequency for a period of twenty minutes, after which the contents of the lusteroid cup were transferred to a high speed centrifuge and spun at 15,000 r.p.m. for one-half hour. The supernatant, which was slightly opalescent, was diluted with an additional 10 ml. of infusion broth and then used in the adsorption tests.

The bacterial cells used in the tests were prepared as described in Materials and Methods. Two strains were used, D43 and D/Pa. Three tubes containing packed D43 cells and three containing packed D/Pa cells where placed in a rack. To one tube of each set were added 10 ml. of Pa bacteriophage titering 9×10^9 phage particles per ml. After allowing one-half hour for adsorption of the virus, the suspensions were centrifuged and the supernatant decanted.

The radioactive medium prepared by disintegrating bacteria in the Magnetostrictor was divided into four equal portions of 5 ml. each. The contents of one tube of D43 treated with bacteriophage and the contents of one tube of D/Pa treated with bacteriophage were each suspended in one of the 5 ml. portions of this radioactive medium. The contents of one tube of untreated cells of each strain were likewise mixed with 5 ml. portions of the same medium. Each of the remaining tubes of D43 cells and D/Pa cells was mixed with 5 ml. of lysate prepared by the use of bacteriophage. All the mixtures were allowed to stand one-half hour and then centrifuged at 3,000 r.p.m. for ten minutes. The supernatants were decanted and the sedimented cells washed six times with saline. The final sediments were transferred to slides for assay of radioactivity.

The results of this experiment are presented in Table 5 and are discussed below.

TABLE 5

CT./MIN.
MAGNETOSTRICTOR LYSATE (M. L.) 105
PHAGE LYSATE (P. L.) 92

Preliminary treatment of cells	*Cells suspended in M.L. and then washed*	*Ct./min.*	*Cells suspended in P.L. and then washed*	*Ct./min.*
Treated with phage	D43	10 ± 2.8		
None	D43	7 ± 2.6	D43	76 ± 5.5
Treated with phage	D/Pa	10 ± 2.8		
None	D/Pa	13 ± 2.9	D/Pa	73 ± 5.3

$\pm$ values refer to standard deviation.

Summary and discussion of experiments of Section I

Examination of Tables 1, 2, and 3 shows that when given amounts of non-metabolizing bacteria were suspended in radioactive, inorganic-phosphate solution assaying from 6,000 to 18,000 ct./min. per 0.1 ml., phosphates were adsorbed by the bacteria to give counts ranging from 82 to 176 ct./min. However, according to the results shown in Table 5, when the suspending medium contained radioactive phosphates assaying at 105 ct./min. per 0.1 ml. resulting from the mechanical disruption of radioactive bacteria, phosphates were adsorbed to give counts ranging from 7 to 13 ct./min. Comparable counts were adsorbed from solutions of inorganic phosphates assaying at 317 to 1,500 ct./min. On the other hand, lysates prepared through the action of bacteriophage on bacteria which had previously been allowed to grow and metabolize in the presence of radioactive phosphates seem to have contained relatively much larger amounts of adsorbable radiophosphorus compounds (Tables 3, 4, and 5). These lysates assayed at 77 to 92 ct./min. per 0.1 ml., and the bacteria allowed to adsorb phosphates from this media assayed at 67 to 91 ct./min. This amount adsorbed corresponds to the amounts adsorbed by similar quantities of bacteria from inorganic solutions of radioactive phosphates assaying at approximately 6,000 to 13,000 ct./min. per 0.1 ml. That these differences were not due to the influence of bacteriophage on the ability of non-metabolizing bacteria to adsorb radiophosphorus compounds was indicated by the experiments whose results are presented in Tables 1, 2, and 5. In these experiments, the presence of bacteriophage did not influence the amount of radiophosphorus taken up by bacteria, either of a phage-sensitive strain or of a phage-resistant strain, whether these bacteria were heat-killed or merely prevented from growing by being kept cold.

The data presented in Tables 4 and 5 further indicate that the radiophosphorus compounds present in lysates of radioactive bacteria prepared through the action of bacteriophage are not all associated with the latter, since bacteria of strains which did not adsorb phage acquired just as much radioactivity as did bacteria which adsorbed 90% of the phage present in suspension. Furthermore, most of the radiophosphorus compounds present in the lysates in these experiments were not adsorbed, either by bacteria which did or by those which did not adsorb bacteriophage.

These experiments indicate, then, that in suspensions of bacteria lysed through the action of bacteriophage there are present phosphorus-containing compounds other than those directly associated with the bacteriophage, which are not present in the inorganic phosphate solutions used in the

experiments and which are not present in normal living bacteria, or at least are not liberated from them by mechanised disruption. No attempt was made to elucidate the nature of such compounds. It was clear, therefore, that any radioactivity acquired by bacteriophage in such systems would be measured together with that of components foreign to the phage, whose radioactivity would then in effect be masked.

In subsequent experiments, differential centrifugation as well as dialysis were used to eliminate, in so far as possible, contaminating radioactive components, in order to allow the activity of the radiophosphorus acquired by bacteriophage to be determined.

Section II

Studies on the Source of Phosphorus in Bacteriophage

In this group of experiments an attempt was first made to determine whether compounds of phosphorus, existing in the bacterial cells prior to infection with bacteriophage, could serve as a source of phosphorus for bacteriophage. Further experiments were then carried out to compare the relative amount of phosphorus in bacteriophage derived from the forementioned source with the amount derived from the medium in which the bacteria and the bacteriophage were suspended.

Experiments 1, 2, and 3

Nine ml. of sterile infusion broth were pipetted into a sterile tube, and one ml. of radioactive Na_2HPO_4 solution was added. The resulting medium was inoculated with D43 and incubated at 37° C. After eighteen hours the cells of the resulting culture were sedimented by centrifuging and washed six times in infusion broth. After the final washing, the cells were resuspended in 20 ml. of infusion broth, and 0.1 ml. of stock Pα was added. The resulting mixture was incubated at 37° C. for one and one-half hours, when lysis was complete. Bacterial debris was removed by centrifugation, and the clarified lysate was transferred to a high speed centrifuge. This centrifuge was an International Centrifuge, No. 1, type SB, with a multispeed attachment and a built-in refrigerating unit, and was operated at 16,000 r.p.m. It was found that 95 to 99% of the virus could be sedimented at this speed and recovered. The supernatant fluids were decanted and the remaining pellets taken up in sterile 0.85% saline solution. The resulting suspension was again centrifuged at 3,000 r.p.m. in an angle head centrifuge and the supernatant again transferred to the high speed centrifuge. This process was repeated through four cycles of differential centrifugation. The final suspension, which was colorless and only slightly opalescent, was transferred to a sac made of dialyzing cellophane and dialyzed against running water at 4° C. for seventy-two hours. By the end of this time, a light precipitate appeared,

which was removed by centrifuging at 3,000 r.p.m. The sediment was tested for its phage content, which was found to be less than 1% of the phage present in the supernatant and was therefore discarded.

The supernatant, the volume of which was 20 ml., was divided into two equal portions of 10 ml. each. To one portion were added packed D43 cells, approximately 1×10^{11} in number, which had been washed in physiological saline. To the other portion were added a like number of packed, washed D/Pα cells. Adsorption was allowed to proceed for approximately thirty minutes, after which the cells were sedimented by centrifuging. The sediment was washed six times in saline and finally spread on slides for assay of radioactivity. On the following day, when the results of the phage titrations became known, it was noted that no phage had been removed from the suspensions either by D43 or by D/Pα cells. The above adsorption procedure was repeated, with the same results.

To each of the supernatants resulting from the second adsorption was added 1.0 ml. of infusion broth, and adsorption was allowed to take place for a third time. The supernatant resulting from the sedimenting of the third mixture of purified lysate and D/Pα cells was mixed with D43 cells and allowed to stand for thirty minutes. All mixtures were tested as above, that is, the suspensions were centrifuged and the sedimented cells washed six times and then spread on a slide for assay of radioactivity. The supernatants from all adsorption mixtures were tested for phage content and radioactivity.

The results of this experiment are summarized in Table 6. It is of interest to note that some adsorption factor seems to be necessary in order that phage Pα be adsorbed by bacteria of a sensitive strain. Previous to this work, only certain members of the "T" phages have been reported to require an adsorption factor, which was found by T. F. Anderson to be tryptophane.[1] No attempt was made in the present study to determine the nature of the adsorption factor described above.

Four cycles of differential centrifugation followed by dialysis removed approximately 97% of the radioactivity of the lysate prepared through phage action on radioactive bacteria.

When radioactive lysates, purified as described above, were mixed with washed bacteria, no adsorption of bacteriophage took place, whether the bacteria used were of phage-sensitive or of phage-resistant strains. At the same time, very little radioactivity was taken up by these bacteria. When a small amount of infusion broth was mixed with the purified lysate, the phage-sensitive bacteria adsorbed approximately 90% of the bacteriophage as well as some radioactivity, whereas the phage-resistant strain did not adsorb a significant amount of bacteriophage and acquired much less radioactivity.

TABLE 6

Assays of radioactivity		*Phage titers*	
	Ct./min.	Original lycate	9.0×10^9
Medium	157,000	After 4th centrifuging	8.8×10^9
Original lysate	2,010	After dialysis	8.5×10^9

Lysate purified by centrifugation

Times centrifuged	*One*		*Two*		*Three*		*Four*	
Portion of centrifuged lysate assayed	*Supernatant*	*Pellet suspension*	*Supernatant*	*Pellet suspension*	*Supernatant*	*Pellet suspension*	*Supernatant*	*Pellet suspension*
Counts/minute	1820	125	35	100	19	83	4	82

Purified lysate mixed with cells

Times mixed	*One*		*Two*		*Three*		*Four*
Types of cells used in mixture	D43	D/Pa	D43	D/Pa	D43	D/Pa	D43
Counts/minute	10	11	12	8	153	14	169
Phage titer of supernatant	7.1×10^9	7.5×10^9	8.1×10^9	7.6×10^9	7.8×10^9	6.9×10^9	6×10^9

The above experiment was repeated twice (Experiments 2 and 3) under identical conditions, using the same solution of radioactive phosphate, and the results were almost identical in each case after correction was made for radioactive decay and for a uranium glass standard count of 360 ct./min. Since both phage-sensitive and phage-resistant strains of bacteria took up little radioactivity under conditions in which neither strain adsorbed bacteriophage, whereas phage-sensitive bacteria took up much more radioactivity under conditions in which bacteriophage was adsorbed, it may be concluded that the radioactivity adsorbed under the latter conditions was directly associated with the bacteriophage particles. This indicates that compounds of phosphorus, present in bacteria and existing prior to infection with bacteriophage, can serve as a source of phosphorus for the latter. However, these experiments do not show whether bacteriophage can also utilize phosphorus compounds synthesized by the bacteria after infection or whether bacteriophage in the presence of sensitive bacteria can utilize phosphates existing in the medium in which the bacteria and the virus are suspended. The following group of experiments was designed to test the above possibilities.

Experiments 4, 5, and 6

Except as otherwise stated, the procedures described below were followed in each of Experiments 4, 5, and 6.

To each of three tubes, labeled *1*, *2*, and *3*, were added 9 ml. of sterile infusion broth. One ml. of radioactive H_3PO_4 solution was pipetted into tubes *1* and *3*, and 1 ml. of saline was pipetted into tube *2*. Each tube was inoculated with a drop of a twenty-four-hour infusion broth culture of D43 and placed in the 37° C. incubator. Eighteen hours later, the cells in tube *1* were sedimented by centrifuging and washed six times in sterile infusion broth. The washed cells were finally suspended in 19 ml. of infusion broth with the addition of 1 ml. of saline and 0.1 ml. of stock bacteriophage Pα. Into tube *2* were pipetted 9 ml. of infusion broth, 1 ml. of radioactive phosphate solution, and 0.1 ml. of stock bacteriophage Pα. Into tube *3* were pipetted 9 ml. of infusion broth, 1 ml. of saline, and 0.1 ml. of stock bacteriophage Pα. These mixtures were placed in the 37° C. incubator for one and one-half hours, at the end of which time lysis was complete. The lysates were clarified by centrifugation and the supernatants decanted. The bacteriophage present in the suspensions was then sedimented in the high speed centrifuge and the supernatants discarded. The pellets were suspended in phosphate buffer and again centrifuged at 3,000 r.p.m. This procedure of low speed centrifugation followed by high speed centrifugation was repeated twice more, the pellets being resuspended each time in fresh sterile phosphate buffer. The final pellet suspensions were removed to dialyzing cellophane sacs and dialyzed against running water at 4° C. for eighty-four hours. At the end of that time, the dialyzed lysates were centrifuged at 3,000 r.p.m. for fifteen minutes to remove precipitated material and the supernatants decanted. The volume of each lysate was made up to 50 ml. with sterile infusion broth.

Each of the purified Lysates *1*, *2*, and *3* was divided into two equal portions of 25 ml. each. One portion of Lysate *1* was added to a tube containing packed D43 cells approximately 1×10^{11} in number, and the other portion was added to a tube containing a like amount of packed D/Pα cells. Lysates *2* and *3* were treated in an identical manner. The cells were suspended by thorough mixing with the aid of pipettes and the suspensions allowed to stand one-half hour. After this time the cells were sedimented by centrifuging and the supernatants decanted into tubes containing fresh-packed cells, but in reverse order, *i.e.*, the supernatant resulting from the centrifugation of a mixture of lysate and packed D43 cells was decanted into a tube containing packed D/Pα cells, and the supernatant resulting from the centrifugation of a mixture of lysate and packed D/Pα cells was decanted into a tube containing packed D43 cells. These mixtures were treated in the same manner as the former ones. The cells which were thrown down by centrifugation of the mixtures were washed six times in saline and finally transferred to slides for assay of radioactivity.

In Experiment 6, reversal of the order of adsorption was carried out a third time. That is, lysates which had been exposed to D43 and D/Pα cells in that order were mixed again with D43 cells, and lysates which had been exposed to D/Pα and D43 cells in that order were mixed again with D/Pα cells.

The results of Experiments 4, 5, and 6 are summarized in Table 7.

TABLE 7

Materials assayed	*Count/Minute*		
	a	*b*	*c*
Medium	275,000	285,000	284,600
Crude lysate 1	5,225 ± 41.7	5,560 ± 43.0	4,760 ± 39.9
Purified lysate 1	58 ± 4.4	135 ± 6.7	46 ± 3.4
Purified lysate 2	36 ± 3.5	54 ± 4.2	35 ± 3.4
Purified lysate 3	46 ± 3.9	171 ± 7.6	51 ± 4.1

	Bacteriophage Titers x 10^8					
	Crude Lysates			*Purified Lysates*		
	a	*b*	*c*	*a*	*b*	*c*
Lysate 1	40	90	150	41	100	200
Lysate 2	45	96	170	44	89	240
Lysate 3	34	110	140	40	90	200

Strain of Cells	*Lysate No.*	*First Adsorption* — *Washed Cells Ct./Min.*			*Supernatant Ct./Min.*			*Bacteriophage Titer after Adsorption x 10^8*		
		a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
D43	1	1790	2285	1530	50	132	41	3.5	19.0	19.0
D/Pa	1	210	580	208	47	151	38	40.0	72.0	200.0
D43	2	495	430	278	33	50	30	3.4	18.0	21.0
D/Pa	2	147	200	246	37	50	35	43.0	65.0	220.0
D43	3	1810	2362	1670	38	172	47	3.6	9.3	18.0
D/Pa	3	114	520	191	43	168	49	35.0	69.0	190.0
		Second Adsorption								
D43	1	3200	2010	1310	48	128	32	3.7	18.0	18.0
D/Pa	1	60	530	154	50	133	35	4.2	20.0	17.0
D43	2	589	412	266	31	52	36	3.0	18.0	16.0
D/Pa	2	48	236	222	32	47	32	3.3	15.0	19.0
D43	3	3090	2082	1340	39	164	39	3.1	20.0	16.0
D/Pa	3	54	510	154	40	168	46	3.5	13.0	18.0
		Third Adsorption								
D43	1			104						15.0
D/Pa	1			92						16.0
D43	2			81						
D/Pa	2			93						
D43	3			72						14.0
D/Pa	3			88						16.0

Counts given in this table are corrected for a uranium glass standard count of 360 ct./min. and for radioactive decay.

Bacteriophage titers given are corrected to correspond to the original volume of lysate.

a = results of Experiment 4; b = results of 5; c = results of 6.

The lysates used in these experiments were of three types: Lysates No. 1 were prepared through the action of bacteriophage on cells which had previously been grown in radioactive media; Lysates No. 2 were prepared through the action of bacteriophage on bacteria which were not radioactive but which were suspended in radioactive media at the time of infection; Lysates No. 3 were prepared from bacteria which were grown in radioactive media and remained suspended in those media at the time of infection with bacteriophage.

The lysates were purified by differential centrifugation and dialysis, after which they were mixed with both phage-sensitive and phage-resistant cells. The phage-sensitive bacteria removed 90% of the bacteriophage activity from these lysates and acquired three to ten times as much radioactivity as did the phage-resistant cells, which did not remove a significant amount of bacteriophage from suspension. Approximately four to six times as much radioactivity was taken up by phage-sensitive cells from the lysates prepared from radioactive bacteria as from the lysates prepared from non-radioactive bacteria suspended in radioactive media.

Following the above series of adsorptions, both series were carried out with the same samples of the lysates, in which the order of adsorption was reversed. Again the phage-sensitive cells removed 90% of the bacteriophage present in the lysates and at the same time adsorbed much more radioactivity than did the phage-resistant cells, which did not take a significant amount of phage out of suspension. Here, too, approximately four to six times as much radioactivity was taken up by phage-sensitive cells from lysates derived from radioactive cells as from lysates derived from non-radioactive cells suspended in radioactive media.

When the order of adsorption was reversed for a third time, *i.e.*, after 90% of the bacteriophage had been removed from all samples of the lysates, the amount of radioactivity taken up by the phage-sensitive bacteria was considerably reduced and now equalled the amount taken up by the phage-resistant cells. In other words, whenever conditions were such that no bacteriophage was adsorbed, a relatively small amount of radioactivity was acquired by the adsorbing cells; and when bacteriophage was adsorbed, a much larger amount of radioactivity was acquired. This seems to confirm earlier conclusions, namely, that the difference between the amount of radioactivity taken up by phage-sensitive cells and phage-resistant cells represents radioactive phosphorus directly associated with the bacteriophage in suspension. However, the major part of the radioactive phosphorus present

in the purified lysates was not removed when 90% of the bacteriophage was removed from suspension. This indicates that the majority of the radiophosphorus in such lysates was not associated directly with bacteriophage and that the methods used were not adequate to purify the bacteriophage completely. That considerable purification of the lysates took place through centrifugation and dialysis is shown by the fact that almost 99% of the radioactivity present in the crude lysates prepared from radioactive bacteria suspended in non-radioactive media is lost by these methods without appreciable loss of bacteriophage activity.

Experiment 7

In general, the procedures followed in this experiment were similar to those in the three preceding ones. However, prior to testing the radioactivity acquired by the bacteriophage by means of adsorption to phage-sensitive cells, a series of six preliminary adsorptions, using phage-resistant cells, was performed on each sample of the lysates. This was done in an attempt to remove the contaminating, non-specifically adsorbing radioactive components which were not eliminated by differential centrifugation and dialysis of the bacteriophage lysate. The results of this experiment are summarized in Table 8.

From the data presented in this table, it can be seen that there was considerable loss of bacteriophage during the purification procedures prior to the preliminary adsorptions with D/Pα cells. Preliminary adsorption by bacteria of strain D/Pα did not remove a significant amount of radioactivity or bacteriophage. Following these adsorptions, bacteria of strain D43 were mixed with the lysates and were found to remove 90% of the remaining bacteriophage and to acquire a significantly larger amount of radioactivity than did the cells of a phage-resistant strain. However, the major portion of radioactivity still remained in the purified lysates after all the adsorption procedures.

More radioactivity was taken up by D43 bacteria from lysates derived from radioactive bacteria than from a lysate derived from non-radioactive bacteria suspended in radioactive medium. Furthermore, the amount of radioactivity acquired by D43 from lysates derived from radioactive bacteria suspended in a non-radioactive medium approximately equalled the amount taken up from the lysates derived from radioactive bacteria suspended in a radioactive medium.

TABLE 8

Assay of radioactivity		*Phage titers*		
Material assayed	*Ct./min.*		*Before purification*	*After purification*
Medium	275,000			
Purified lysate 1	65 ± 4.7	Lysate 1	5.8×10^9	1.7×10^9
Purified lysate 2	25 ± 2.9	Lysate 2	7.2×10^9	1.8×10^9
Purified lysate 3	81 ± 5.2	Lysate 3	6.0×10^9	1.5×10^9

Adsorption No.	*Strain of cells*	*Lysate No.*	*Radioactivity of washed cells ct./min.*	*Radioactivity of supernatant ct./min.*	*Phage titer after adsorption*
	D/Pa	1	61		
1	D/Pa	2	59		
	D/Pa	3	54		
	D/Pa	1	53		
2	D/Pa	2	55		
	D/Pa	3	61		
	D/Pa	1	38		
5	D/Pa	2	33		
	D/Pa	3	43		
	D/Pa	1	29	67	1.0×10^9
6	D/Pa	2	23	22	1.1×10^9
	D/Pa	3	22	72	8.0×10^8
	D43	1	201	63	1.1×10^8
7	D43	2	34	23	1.0×10^8
	D43	3	204	65	1.2×10^8

Counts given were corrected for a U. G. count of 360 and for radioactive decay of from 0.85 mc/ml. stock solution to 0.25 mc/ml.

Discussion

Various investigators have, in the past, used differential centrifugation and dialysis to obtain purified bacteriophage[6, 18, 26] and have then performed chemical analyses on the material so obtained. Although chemical and physical determinations of homogeneity were carried out and the materials obtained were shown to be more or less pure according to these standards, it was felt that substances other than bacteriophage may have been present which were indistinguishable from the latter through ordinary chemical and physical tests. Therefore, biological methods in combination with the usual physical methods for detection of radioactive tracer elements were used in this work rather than the less sensitive chemical methods. The results of

such experiments confirmed the suspicion that differential centrifugation and dialysis did not eliminate all contaminating substances. These procedures were found to eliminate approximately 95% of the phosphorus compounds present in the crude lysate, but, when 90% of the bacteriophage was removed from the resulting "purified" bacteriophage suspension by means of specific adsorption with a sensitive strain of cells, it was found that most of the phosphorus compounds still present after centrifugation and dialysis still remained behind. This seems to indicate that most of the material remaining in suspension in the lysates purified by the above methods was not bacteriophage.

A certain degree of error was introduced by the fact that not all of the P^{32} taken up by cells suspended in radioactive lysates was part of the bacteriophage adsorbed by the cells. This was shown by the fact that cells which were not capable of adsorbing bacteriophage did acquire some radioactivity and that the phage-sensitive strain of bacteria, in the absence of the co-factor necessary for phage adsorption, likewise gained some radioactivity in an amount approximately equal to that shown by the phage-resistant cells. However, when a medium containing the co-factor was added to the above system, phage-sensitive cells picked up much more radioactivity than in the absence of the co-factor, indicating that the difference was due to the radioactive phosphorus contained in the adsorbed bacteriophage particles. That this difference was not due to differences in the ability of phage-sensitive and phage-resistant strains of bacteria to adsorb pho- -te or to increased adsorbing ability of the bacteria due to the presence of bacteriophage was also shown in Section I of the experimental work.

It was also found that adsorption of bacteriophage by phage-sensitive bacteria could not be performed directly from the unpurified lysates for the purpose of measurement of the P^{32} content of the bacteriophage, since comparatively large amounts of other phosphorus-containing materials were present in the lysate which were adsorbed to the bacteria at the same time as the phage particles themselves were adsorbed. These contaminating materials were adsorbed by phage-resistant strains of organisms as well as by the phage-sensitive strain, and therefore no conclusion could be drawn as to the amount of P^{32} contained by the bacteriophage particles themselves. When the lysates were partially purified by the methods mentioned above, most of the non-specifically adsorbing radiophosphorus compounds were eliminated.

It is of interest to note that lysates of bacteria where lysis was induced by bacteriophage contain compounds, other than bacteriophage, qualita-

tively different from those present in normal bacteria which were lysed by mechanical means. This is indicated by the fact that, when phage-resistant strains of bacteria were added to lysates entirely comparable in their content of P^{32} but produced by bacteriophage on one hand and by mechanical disruption on the other, the added bacteria adsorbed much more radioactivity from the former lysate than from the latter. No attempt was made to determine the cause of this difference.

After it was established that bacteriophage could obtain phosphorus from compounds contained in the bacterial cells, the question arose as to whether this was the main source or whether the medium could satisfy part or all of the phosphorus requirements of the bacteriophage. In order to resolve this question, three types of bacteria-bacteriophage systems were used:

1. Bacteria were allowed to metabolize in the presence of radio-phosphates. The cells were subsequently washed and suspended in a medium free from radioactivity and then infected with virus. After lysis occurred, the bacteriophage which had been grown under these conditions was tested for the presence of P^{32}. In this system, the only source of P^{32} for the bacteriophage consisted of P^{32} contained in the bacterial cells prior to infection with virus.

2. Non-radioactive bacteria were suspended in a medium containing P^{32} and were then infected with virus. The chief source of P^{32} for the bacteriophage here, of course, consisted of compounds present in the medium. During the period prior to lysis, the bacteria did have an opportunity to incorporate P^{32} which the virus may have subsequently been able to utilize, but control experiments not given in the report showed that the amount acquired by the bacteria in the time prior to lysis could not have been over one-fourth the amount obtained by the cells used in system 1.

3. Radioactive bacteria prepared as in system 1 were allowed to remain in medium containing P^{32}. These cells were infected with virus, and therefore the bacteriophage had the opportunity to obtain P^{32} from both sources, the cells and the medium.

After the virus had been grown in the above three sets of conditions, it was found that virus grown under the conditions in systems 1 and 3 contained much more radioactivity than did virus grown as in system 2. Furthermore, the radioactivity of virus grown as in system 1 equalled that of virus grown as in system 3. These results indicated that the bacteria alone furnished the phosphorus compounds for the virus particles. If the medium had furnished any phosphorus, virus grown as in system 3 would have contained more P^{32} than that grown as in systems 1 or 2. If the medium furnished the major portion or all of the phosphorus contained in the virus,

the bacteriophage grown under the conditions of system 2 would have contained more radioactivity than the virus resulting from system 1 but would have been approximately equal in P^{32} content to the virus resulting from system 3. The fact that bacteriophage grown in non-radioactive cells suspended in radioactive medium contained some radioactivity might be explained by the fact that, prior to lysis, the bacteria in this system did have some opportunity to grow and metabolize P^{32} compounds which were subsequently utilized by the bacteriophage.

These results seem to conflict with those obtained by Cohen[3, 4, 5] and by Putnam and Kozloff.[25] These investigators, working with r^+ viruses of the "T" series, found that the main source of phosphorus for bacteriophage was to be found in the medium rather than in preformed compounds of phosphorus existing in bacteria prior to infection. There seem to exist several possible explanations for the discrepancies between the results of the above authors and those of the present investigations: (a) The investigations of Cohen and of Putnam and Kozloff were carried out on so-called "lysis inhibiting" strains of bacteriophage described by Hershey[16, 17] and by Doermann.[10] No turnover in ribonucleic acid was found to accompany the synthesis of desoxyribonucleic acid during the growth of bacteriophage. However, working with other viruses, many workers have found this not to be the case. Gratia, Brachet, and Jeener,[15] for example, found that cells of silkworms infected with the virus of silkworm jaundice showed a rapid turnover of ribonucleic acid accompanying the synthesis of desoxyribonucleic acid. Thus, the host cells may have furnished the materials necessary for formation of the desoxyribonucleic acid of the virus. This may have been the case in the present investigations, where a strain of bacteriophage was used which did not belong to the lysis inhibiting group. It therefore seems possible that bacterial viruses of other than lysis inhibiting strains have the ability to organize bacterial enzymes in such a manner as to bring about synthesis of viruses, using preformed bacterial compounds as a substrate. (b) Differential centrifugation and dialysis were used by the above-mentioned authors to purify their bacteriophage preparations. These procedures seem to have been adequate to give preparations showing chemical and physical homogeneity, but, in view of the present findings, the preparations may not have been biologically homogeneous. (c) In the investigations presented here, the concentration of cells infected by bacteriophage was fairly high. The resulting lysate showed an average yield of virus of three to five bacteriophage particles per host cell. In the work of Cohen and of Putnam and Kozloff, the average yield per cell was twenty to thirty

virus particles. When low concentrations of bacterial cells are used and infection of these cells takes place in optimal media, it is not unusual to obtain burst sizes ranging from one hundred to three hundred per cell. Thus it may well be that with changes of environment, corresponding changes in the metabolic processes occur which can determine the source of materials used in the growth of bacteriophage.

Conclusions

1. The presence of bacteriophage does not influence the adsorption of phosphates by non-metabolizing bacteria.

2. During the course of growth of bacteriophage and lysis of the host cells, compounds of phosphorus are formed which are not directly associated with the bacteriophage particles and which differ in nature from those found in normal cells.

3. The usual methods of purifying bacteriophage by differential centrifugation and dialysis are not always adequate for the purification of bacteriophage.

4. Under conditions where the burst size is small, the source of phosphorus of the bacteriophage described in this work is to be found in phosphorus compounds existing in bacterial cells prior to their infection with bacteriophage.

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THE DISAPPEARANCE OF GLUCOSE FROM INCUBATED BLOOD CONTAINING ADDED ELECTROLYTES*†

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It has been demonstrated that the disappearance of glucose from defibrinated human blood during incubation at 37° C. is associated with transfers of ions between plasma and cells.[2, 7, 12, 13] This raises the possibility that the rate of this loss of glucose may be influenced by altering the concentrations of electrolytes present in such a system. The likelihood that such an interrelationship exists is supported by the demonstration that ions serve as catalysts in the glycolytic cycle of animal tissues and of yeasts.[5] The studies in this paper, in part presented in a preliminary note elsewhere,[3] describe the influence of various inorganic salts of sodium and of certain inorganic chlorides upon the rate at which glucose disappears from incubated blood.

Procedure

Venous blood withdrawn from healthy adults and diabetic or non-diabetic patients was defibrinated aerobically by stirring with a glass rod during a six- to eight-minute period. Most of the subjects were in the absorptive state. Following the addition of enough of a 25% solution of glucose to raise the concentration of sugar to 159 mg.% or higher, the blood was apportioned in 2.0 or 5.0 cc. aliquots into 25 cc. Erlenmyer flasks.

In the series of experiments presented in Table 1 solutions of NaCl, KCl, or $MgCl_2$ in water, 4.0, 2.0, and 0.25 equivalents per liter, were introduced in volumes sufficient to increase the concentration of these ions in the blood samples by 40, 20, and 2.5 milliequivalents per liter respectively. In another group, Table 2, the effects of similar increases of $CaCl_2$, NH_4Cl, and HCl were studied. Table 3 describes changes in the rate of glucose disappearance induced by the addition of Na_2SO_4, Na_2HPO_4, $NaHCO_3$, or NaOH. In the final series of experiments, Table 4, alterations produced by increments of NH_4Cl, Na_2HPO_4, or $(NH_4)_2HPO_4$ have been compared.

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Following the addition of the aqueous solutions of these various salts, and of distilled water only to the control bloods to maintain constant volumes, the Erlenmyer flasks were tightly stoppered and then incubated at 37° C., with occasional shaking, for seven hours. The concentrations of blood glucose were measured in duplicate before and after incubation.[1, 28] Control studies indicated that the addition of the various salts did not interfere with the analytical procedure for glucose, and that the results presented in these tables cannot be attributed to bacterial contamination.

Results

In the first twelve experiments presented in Table 1 it is evident that the rate of glucose loss was entirely unaffected by the addition of the chloride

TABLE 1

DISAPPEARANCE OF GLUCOSE FROM INCUBATED BLOOD CONTAINING ADDED NaCl, KCl, or $MgCl_2$

Expt.	*Bl. sugar at start** (*mg. %*)	*Level of sugar (mg.%) after incubation of blood containing added salt in following concentrations*			
		0 mEq./L.	*2.5 mEq./L.*	*20 mEq./L.*	*40 mEq./L.*
1. NaCl	244	164	162	164	162
2. NaCl	375	326	316	318	326
3. NaCl	163	78	82	80	79
4. NaCl	200	83	84	83	89
5. NaCl	291	214		210	210
6. NaCl	377	308	300	306	300
7. KCl	248	185	180	180	184
8. KCl	229	159	149	152	153
9 KCl	223	147	139	140	141
10. KCl	264	168	164	169	164
11. KCl	247	175	161	170	171
12. KCl	248	168	164	176	172
13. $MgCl_2$	159	95	94	105	106
14. $MgCl_2$	196	115	107	116	117
15. $MgCl_2$	211	84	81	93	94
16. $MgCl_2$	248	136	132	144	146
17. $MgCl_2$	193	120	128	122	117
18. $MgCl_2$	225	135	147	137	169

* Following the addition of a solution of 25% glucose in water. Similar procedure employed in experiments in Tables 2, 3, and 4.

salts of sodium or potassium in amounts sufficient to increase their concentration in blood by as much as 40 mEq. per liter. The maximum variations from the controls observed in this series, Experiments 4 and 11, are

not significant in view of the error of about 5% inherent in the measurement of the blood glucose levels. Similar increments of $MgCl_2$, on the other hand, definitely retarded glucose loss in one experiment (Number 18 in Table 1), and apparently exerted a similar though less pronounced effect in two others (Numbers 13 and 15). Since the final blood glucose concentration in these three studies was higher than that of the controls by 10% or more, these differences are probably significant.

In contrast to the entirely negative results obtained with NaCl or KCl, and the occasional retardation observed with $MgCl_2$, alterations in the disappearance rate of glucose occurred consistently in blood in which $CaCl_2$, NH_4Cl, or HCl had been increased by 40 mEq. per liter (Table 2). It is

TABLE 2

ALTERATION IN THE RATE OF GLUCOSE LOSS IN INCUBATED BLOOD FOLLOWING THE ADDITION OF $CaCl_2$, NH_4Cl, OR HCl

	Expt.	*Bl. sugar at start (mg. %)*	*Level of sugar (mg.%) after incubation of blood containing added salt in following concentrations*			
			0 mEq./L.	*2.5 mEq./L.*	*20 mEq./L.*	*40 mEq./L.*
1.	$CaCl_2$	248	136	134	140	174
2.	$CaCl_2$	163	78	85	96	104
3.	$CaCl_2$	211	84	82	100	114
4.	$CaCl_2$	224	134	134	142	152
5.	$CaCl_2$	196	115	108	124	135
6.	$CaCl_2$	159	95	92	106	120
7.	$CaCl_2$	196	97	101	111	
8.	$CaCl_2$	257	161	173	195	
9.	NH_4Cl	224	128	118	97	102
10.	NH_4Cl	227	163	149	138	137
11.	NH_4Cl	273	182	168	144	146
12.	NH_4Cl	229	159	150	132	131
13.	NH_4Cl	248	185	170	164	160
14.	HCl	227	163	162	203	222
15.	HCl	236	156	162	202	231
16.	HCl	248	185	180	214	245
17.	HCl	229	159	164	194	232
18.	HCl	222	141	150	180	214

apparent that the calcium salt or HCl invariably retarded the loss of glucose at these concentrations, while ammonium chloride accelerated the process. With lesser increases, 20 mEq. per liter, similar but less marked changes

were observed in each of the experiments with HCl or NH_4Cl, and in most of the studies with $CaCl_2$. In the blood samples in which the effects of small increments were tested, 2.5 mEq. per liter, no definite or consistent trend was observed with $CaCl_2$ or HCl. NH_4Cl, however, still seemed to accelerate the catabolism of glucose in view of the increased fall in the level of sugar with progressively larger amounts of NH_4Cl.

Acceleration of glucose disappearance was generally observed in blood in which the two higher concentrations of Na_2SO_4, Na_2HPO_4, $NaHCO_3$, or NaOH were present (Table 3). It is to be noted, however, that in the studies with NaOH maximum increases in the rate at which sugar dis-

TABLE 3

ACCELERATION OF GLUCOSE CATABOLISM FOLLOWING THE ADDITION OF SULFATE, PHOSPHATE, BICARBONATE, OR HYDROXYL IONS

	Expt.	*Bl. sugar at start (mg. %)*	*Level of sugar (mg.%) after incubation of blood containing added salt in following concentrations*			
			0 mEq./L.	*2.5 mEq./L.**	*20 mEq./L**	*40 m.Eq./L.**
1.	Na_2SO_4	234	156	108	64	54
2.	Na_2SO_4	185	139	113	99	87
3.	Na_2SO_4	211	139	96	56	46
4.	Na_2SO_4	200	83	83	50	50
	Na_2HPO_4			67	38	30
5.	Na_2SO_4	377	308	300	274	255
	Na_2HPO_4			279	254	232
6.	Na_2SO_4	375	326	314	282	265
	Na_2HPO_4			294	262	232
7.	Na_2SO_4	288	187	165	140	134
	Na_2HPO_4			167	117	100
8.	Na_2SO_4	283	178	153	130	115
	Na_2HPO_4			153	109	79
9.	$NaHCO_3$	227	163	156	146	125
10.	$NaHCO_3$	291	214	216	200	167
11.	$NaHCO_3$	223	147	128	109	93
12.	NaOH	273	182	165	129	168
13.	NaOH	229	159	142	114	128
14.	NaOH	248	185	160	137	140

* Expressed as mEq./L., assuming that these salts dissociate fully. This is not necessarily the case, of course, in a solution of Na_2HPO_4, for example. It may be true, however, if any individual electrolyte is removed from solution following dissociation.

appeared were observed at 20, and not at 40, mEq. per liter. In a series of comparisons in Experiments 4 through 8, Na_2HPO_4 in the two higher concentrations accelerated glycolysis to an extent greater than did Na_2SO_4. A similar quantitative difference was present with the smallest increments in the sense that Na_2SO_4 occasionally had no effect on glycolysis, while Na_2HPO_4 invariably hastened it. The results with $NaHCO_3$ and with NaOH using these same small increments were also variable. Acceleration was observed in some of the experiments and no change in the remainder.

The final table, Number 4, illustrates that the accelerating effects of NH_4Cl and of Na_2HPO_4 are clearly additive. From the drop observed with

TABLE 4

ADDITIVE EFFECTS OF PHOSPHATE AND AMMONIUM IONS IN ACCELERATING THE DISAPPEARANCE OF GLUCOSE FROM BLOOD DURING INCUBATION

	Bl. Sugar at start	*Level of sugar (mg.%) after incubation of blood containing added*			
Expt.	*(mg.%)*	*0 mEq./L.*	*NH_4Cl 40 mEq./L.*	*Na_2HPO_4 40 mEq./L.*	*$(NH_4)_2HPO_4$ 40 mEq./L.*
1.	273	182	146	92	55
2.	222	127	100	37	16
3.	264	168	144	75	49
4.	257	191	171	135	124
5.	236	156	136	80	68
6.	247	175	155	101	81

each salt independently one would expect that the ammonium and phosphate ions in combination would lower the sugar to 56, 10, 51, 115, 60, and 81 mg.%, respectively, in Experiments 1 through 6. This agrees closely with the observed values of 55, 16, 49, 124, 68, and 81 mg.%.

Discussion

Unequivocal evidence has been presented that the addition of certain salts to defibrinated blood can modify the rate at which glucose is converted to lactic acid *in vitro*. These increments of electrolytes produced comparable effects in blood obtained from both diabetic and non-diabetic subjects. In view of the negative findings in the NaCl studies, and the essentially complete ionization of this salt in solution in body fluids, it seems reasonable to assert that increases in the sodium and the chloride ion exert no effect,

or a diametrically opposed one, on the rate of glucose loss. If the former interpretation is correct, the retardation observed with $CaCl_2$, HCl, or $MgCl_2$ is to be attributed to the calcium, hydrogen, and magnesium ions respectively. Similarly, the accelerating effect of NH_4Cl is presumably related to the ammonium ion. Also, if in accordance with the above reasoning the sodium ion is taken to be without effect on the loss of glucose, the hastening of this process by $NaHCO_3$, Na_2SO_4, Na_2HPO_4, or NaOH must be ascribed to the particular ions associated with the sodium. It would appear, therefore, that, except for chloride, the anions which were studied accelerated, while the hydrogen ion and the divalent cations (calcium and magnesium) retarded the breakdown of glucose. The acceleration observed with NH_4Cl under these circumstances would be the only example of an acceleration of glucose loss by a univalent cation.

The mechanisms whereby these salts exert their particular effects are not clear. Although changes in the pH toward the acid or the alkaline side have long been known to be associated with a decrease or an increase in glycolysis respectively,[20, 21, 24] this does not appear to be the sole explanation for the findings in these studies. Direct measurement of the pH by means of a glass electrode at the end of incubation failed to reveal, except in the case of NaOH, HCl, and $NaHCO_3$, any significant deviation from the control specimens. This can perhaps be most clearly illustrated by citing the results of pH measurements in a representative experiment at the end of seven hours of incubation of blood samples containing salts which failed to affect, retarded, or accelerated glucose disappearance. KCl, $CaCl_2$, and $(NH_4)_2HPO_4$ were added to aliquots of blood in triplicate; at the end of incubation the pH values were found to be 7.62, 7.63, and 7.53, respectively. There is no evidence in this study, nor in others comparable to it, that the increased glucose loss following $(NH_4)_2HPO_4$ is to be attributed directly to an upward shift of the pH; nor that the retardation observed after $CaCl_2$ is produced by a shift toward the acid side. The slightly lower values in the $(NH_4)_2HPO_4$ blood may merely reflect the presence of larger amounts of lactic acid as a result of increased glycolysis.[18] Similar findings were obtained in studies in which the pH was measured in bloods containing added NaCl, $MgCl_2$, Na_2HPO_4, Na_2SO_4, or NH_4Cl in 40 mEq. per liter concentrations.[4] In all of these experiments the pH of the blood immediately following the aerobic defibrination was found to be approximately 7.8, presumably as a consequence of the loss of carbon dioxide,[16] and a lowering of its temperature following withdrawal.[25] With the production of lactic acid from glucose, the pH decreased.[8, 17] On the other hand, the effects of

NaOH, HCl, and $NaHCO_3$, are quite likely related to the optimal pH range for the glycolytic process.[18, 21] The complete cessation of glucose catabolism with high concentrations of HCl, and the lesser effects of 40 than of 20 mEq. per liter increases of NaOH in accelerating the process, represent in all probability injury or destruction of blood cells. This is supported by the darker color of blood containing the higher concentrations of HCl and NaOH.

It may be that the alterations observed after addition of the calcium salts were related to hemolysis, since destruction of the blood cells is known to interfere with glycolysis.[6, 9, 15, 19] In a series of separate experiments not reported here it has not been possible to demonstrate retardation following the addition of calcium salts without producing a concomitant hemolysis. Similar results were obtained with another divalent ion, barium, which has the same effect on the disappearance of blood glucose as does calcium and produces an equivalent degree of blood cell destruction.[4] However, even though hemolysis may be a factor in the effects produced by calcium, it is not necessarily the sole reason for the retardation of glucose loss. Calcium might act, for example, by removing from solution as insoluble calcium salts other participants in the cycle, such as phosphates. The studies of Roche and Roche do not clarify this point, since the degree of hemolysis was not noted.[22]

A number of observers have commented upon the acceleration of the blood glycolytic process following the addition of phosphate salts.[10, 14, 22, 23] Mass effects are probably responsible for this increased catabolism of glucose, since the phosphate radicle participates in the intermediate metabolism of this carbohydrate.[5, 10, 12, 27] On the other hand, explanation of the effectiveness of the sodium sulfate is particularly difficult because increments of the sulfate ion are apparently excluded from blood cells.[11] This problem cannot be circumvented by suggesting that the sulfate salt first hemolyzes cells. This does not appear to be the case, even though its concentration is increased by as much as 40 mEq. per liter. It should be pointed out, however, that hemolyzed blood still does respond, though at a lower rate, to the accelerating action of salts which contain the sulfate or phosphate radicle.[4]

These findings provide further evidence of the participation of inorganic ions in one type of carbohydrate catabolism. It is realized that in some, but not all, instances the concentrations at which the effects described in this paper were observed outside the realm of physiological alterations.

Summary and conclusions

1. The disappearance of glucose from defibrinated human blood during incubation may be retarded by $CaCl_2$, $MgCl_2$, or HCl.

$NaHCO_3$, Na_2SO_4, Na_2HPO_4, NaOH, NH_4Cl, or $(NH_4)_2HPO_4$.

3. Increments of NaCl and KCl as high as 40 mEq. per liter fail to modify the rate of glucose disappearance.

2. An accelerated loss of glucose occurs in blood which contains added

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CANCELLATION OF FLUORIDE INHIBITION OF BLOOD GLUCOSE CATABOLISM*†

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The soluble inorganic salts of fluoride are recognized to be highly toxic when administered parenterally or *per os* to animals or humans.[15, 18, 20] When taken in sufficiently large amounts such compounds may prove fatal within a matter of minutes. The fluoride ion appears to be an enzyme poison.[19, 22, 34] It is known, for example, that fluoride added to suitable preparations of tissues or plants *in vitro* will block the enzyme enolase, forming a magnesium-fluoride-phosphate-enolase complex and thereby inhibiting glycolysis at the point where phosphoglyceric acid is converted to phosphopyruvic acid.[10, 11, 35] Soluble inorganic salts of fluoride will also suppress the disappearance of glucose which characteristically occurs in blood on standing, but no data are available as to whether or not this effect is reversible.[3, 4, 9, 17, 30, 32] Such information might well be useful in planning the therapy of fluoride poisoning. The studies herein reported, and presented in preliminary form elsewhere,[5] indicate that in defibrinated blood this fluoride effect can in fact be cancelled by the introduction of certain calcium and magnesium salts.

Procedure

Samples of defibrinated human blood containing added glucose, sodium fluoride, and aqueous solutions of various salts, alone or in combination as recorded in Tables 1 through 5, were incubated in stoppered Erlenmyer flasks for seven hours at 36-37° C., in accordance with the procedure described in an earlier report.[7] The concentration of glucose in each flask was determined prior to and immediately following incubation.[2, 33] The effectiveness of increments of $CaCl_2$ or of $MgCl_2$ in cancelling sodium fluoride inhibition of glucose catabolism was determined in the first two sets of experiments (Tables 1 and 2). Table 3 consists of studies with other chloride salts (sodium, potassium, and ammonium) to establish whether the results in the previous two groups of experiments are to be attributed to the anions or to the cations. In

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the last two sets of studies known accelerators of glucose catabolism in defibrinated blood[7, 13, 16, 20, 21] were tested in attempts to overcome the characteristic effect of sodium fluoride (Tables 4 and 5).

Results

The data in Table 1 indicate that the inhibition of glucose catabolism by fluoride in concentrations of 20 mEq. per liter can be cancelled almost completely, and at times completely, by adding $CaCl_2$ in amounts sufficient to produce a final level of 40 or 20 mEq. per liter. Lesser concentrations of this calcium salt, 2.5 mEq. per liter, had no discernible effect upon the fluoride block. From Table 2 it is apparent that a similar removal of the fluoride effect followed the addition of $MgCl_2$. This was less complete, however, than that produced by $CaCl_2$ since the 20 mEq. increments of the magnesium salt were distinctly less effective than the 40 in permitting the resumption of glucose catabolism. The lowest concentration tested, 2.5 mEq. per liter, was again, as in the $CaCl_2$ studies, totally ineffectual.

From Table 3 it is clear that this partial or complete cancellation of fluoride inhibition by $CaCl_2$ or $MgCl_2$ is to be ascribed to the calcium and the magnesium ions rather than to chloride. The other chloride salts tested (NaCl, KCl, NH_4Cl) in no instance modified the fluoride block. It is to be noted that this was true even though one of the salts tested, NH_4Cl, moderately accelerated glucose loss in the control blood (final column, Table 3). Attempts to break through the fluoride block by introducing other salts known to accelerate glucose loss[6, 7] were similarly without success. Thus, in six experiments in Table 4, $NaHCO_3$, Na_2SO_4, or Na_2HPO_4 added in concentrations higher than those of fluoride did not cause a resumption of glucose catabolism. $(NH_4)_2HPO_4$ proved similarly ineffective in four of the experiments. The data presented in Table 5 do indicate, however, that once glucose catabolism has been restarted in fluoride-treated blood by introducing a magnesium salt, the accelerating effect of the sulfate ion can again be demonstrated. In five experiments the blood sugar had dropped much further in NaF-$MgSO_4$ blood than in samples which contained NaF and $MgCl_2$. Moreover, in four of these five instances the rate of glucose catabolism exceeded that of the controls containing only water introduced to maintain final volumes constant and no added electrolytes. The Na_2SO_4 and NaCl studies in this table again indicate the ineffectiveness of these salts against the fluoride block, and serve as controls for the sulfate and chloride ions introduced as $MgSO_4$ and $MgCl_2$.

TABLE 1

CANCELLATION OF SODIUM FLUORIDE EFFECT BY $CaCl_2$

			Level of sugar (mg.%) after incubation of blood containing added				
			NaF: 20 milliequivalents per liter				
Expt.	Bl. sugar at start (mg.%)	H_2O only	H_2O	$CaCl_2$: 40 mEq./L.	$CaCl_2$: 20 mEq.	$CaCl_2$: 2.5 mEq./L.	$CaCl_2$: 40 mEq./L. only
1.	202	153	200	160	155	196	163
2.	184	118	174	132	134	170	136
3.	224	142	214	169	163	218	169
4.	236	159	222	174	179	224	180
5.	201	115	191	141	136	186	135
6.	217	132	197	156	156	201	156
7.	251	174	262*				

* Introduction of $CaCl_2$ 20 mEq./L. to this sample at end of seven hours' incubation was followed by only a slight decrease in blood sugar to 237 mg.% during an additional seven hours at 37° C. During this same period the glucose in the control sample fell from 174 to 100 mg.%. Failure to cancel the fluoride effect was also observed following the delayed administration of $MgCl_2$ in this same experiment.

TABLE 2

LOSS OF SODIUM FLUORIDE EFFECT FOLLOWING THE ADDITION OF $MgCl_2$

			Level of sugar (mg.%) after incubation of blood containing added				
			NaF: 20 milliequivalents per liter				
Expt.	Bl. sugar at start* (mg.%)	H_2O only	H_2O	$MgCl_2$: 40 mEq./L.	$MgCl_2$: 20 mEq./L.	$MgCl_2$: 2.5 mEq./L.	$MgCl_2$: 40 mEq./L. only
1.	224	128	207	160	171	210	130
2.	263	172	259	210	233	248	183
3.	242	144	224	178	191	227	156
4.	222	158	196	154	171	201	161
5.	222	149	217	168	186	219	163
6.	220	141	214	161	176	209	151

* Blood sugar following the addition of a 25% solution of glucose in water to blood from diabetic and non-diabetic subjects. Similar procedure followed in experiments recorded in Tables II through V.

TABLE 3

FAILURE OF NaCl, KCl, OR NH_4Cl TO MODIFY THE INHIBITORY EFFECTS OF SODIUM FLUORIDE

Expt.	Bl. sugar at start (mg.%)	H_2O only	Level of sugar (mg.%) after incubation of blood containing added: NaF: 20 milliequivalents per liter; H_2O	NaCl: 40 mEq./L.	NaCl: 20 mEq./L.	NaCl: 2.5 mEq./L.	NaCl: 40 mEq./L. only
1.	236	169	204	210	213	208	168
2.	203	122	197	199	203	201	126
3.	201	115	191	192	190	195	115
			NaF: 20 milliequivalents per liter; H_2O	KCl: 40 mEq./L.	KCl: 20 mEq./L.	KCl: 2.5 mEq./L.	KCl: 40 mEq./L. only
4.	291	214	286	273	278	278	213
5.	236	169	204	203	201	218	174
6.	203	122	197	196	200	196	123
			NaF: 20 milliequivalents per liter; H_2O	NH_4Cl: 40 mEq./L.	NH_4Cl: 20 mEq./L.	NH_4Cl: 2.5 mEq./L.	NH_4Cl: 40 mEq./L. only
7.	264	183	259	258	261	261	116
8.	224	128	207	208	214	208	102
9.	227	163	226	220	219	216	137

TABLE 4

FAILURE OF $NaHCO_3$, Na_2SO_4, Na_2HPO_4, OR $(NH_4)_2PO_4$ TO AFFECT THE SODIUM FLUORIDE BLOCK

	Bl. sugar at start	*H_2O only*	*Level of sugar (mg.%) after incubation of blood containing added NaF: 20 milliequivalents per liter*				
Expt.	*(mg.%)*		*H_2O*	*$NaHCO_3$: 40 mEq./L.*	*Na_2SO_4: 40 mEq./L.*	*Na_2HPO_4: 40 mEq./L.*	*$(NH_4)_2PO_4$: 40 mEq./L.*
1.	296	205	259	250	267	270	
2.	222	127	195	197	205	204	
3.	264	168	250	246	246	257	246
4.	257	191	259	248	243	269	261
5.	248	168	236	227	227	254	233
6.	247	175	245	243	247	240	240

TABLE 5

ACCELERATION OF GLUCOSE LOSS BY SULFATE FOLLOWING REMOVAL OF THE FLUORIDE BLOCK BY MAGNESIUM

	Bl. sugar at start	*H_2O only*	*Level of sugar (mg.%) after incubation of blood containing added NaF: 20 milliequivalents per liter*				
Expt.	*(mg.%)*		*H_2O*	*$MgCl_2$: 40 mEq./L.*	*$MgSO_4$: 40 mEq./L.*	*Na_2SO_4: 40 mEq./L.*	*NaCl: 40 mEq./L.*
1.	264	168	250	192	168	246	246
2.	222	127	195	137	117	201	201
3.*	180	90	168	103	69	164	163
4.*	213	139	205	153	112	199	197
5.*	190	112	184	130	101	187	179

* Salts added in amounts sufficient to increase concentration in blood by 80 rather than 40 mEq. per liter.

Discussion

Unequivocal evidence has been presented that the addition to blood of soluble calcium or magnesium salts cancels in part or completely the characteristic effects of fluoride. The usual inhibition of glucose loss by this halide is no longer evident in blood from diabetic or non-diabetic subjects incubated under these circumstances. It is true, however, that the rate of glucose loss was at times lower in fluoride-calcium or fluoride-magnesium samples than in the controls. This slight retardation need not represent, however, a residual fluoride effect. It may well be attributed rather to an inhibitory action by the calcium and magnesium salts themselves. This effect has been demonstrated in previous studies,[7] and is again evident in Tables 1 and 2 upon comparison of the final sugar concentration in control samples with those found in blood to which either calcium or magnesium salts had alone been added.

It is probable that this cancellation of fluoride activity results from the removal of this ion from solution as an insoluble salt, thereby releasing glycolytic enzymes from its influence. This interpretation is in keeping with the finding that of the salts tested only those which can form highly insoluble fluorides permitted the resumption of glucose catabolism. This would explain the greater efficacy of $CaCl_2$ compared to $MgCl_2$ seen in Tables 1 and 2, since the fluoride salt of the former is far less soluble.[14] This point of view is further supported by the observation that (a) $BaCl_2$, which can form the insoluble salt BaF_2, is quite as effective as $CaCl_2$ in cancelling the fluoride effect, and that (b) insoluble fluoride salts (CaF_2, MgF_2, and BaF_2) do not inhibit glucose loss from incubated blood.[8] Finally, it is apparent from the last table that blood mixed with fluoride and then treated with magnesium or calcium salts will respond in a characteristic fashion to the accelerating action of sulfate. This interpretation does not exclude the possibility that, in addition to removing the fluoride ions which suppress the glycolytic enzymes, the calcium or magnesium salts replace the body stores of these cations. This might conceivably be of importance in inducing a resumption of carbohydrate metabolism in view of the demonstrated fact that the magnesium ion serves as a catalyst at one or more points in the glycolytic cycle in tissues.[10] Such a view is at variance with the findings of Warburg and Christian which indicated that *in vitro* the inhibition of purified enolase by fluoride was enhanced by increasing the concentration of magnesium ions.[35] In our own studies, increasing the levels of magnesium ion diminished the fluoride effect. This difference is presumably related to the fact that in the experiments herein reported a

biological fluid rather than a purified enzyme was employed, and the magnesium: fluoride ratios were different. The possibility that the ferment in blood is not identical with the enolase obtained from tissues or plants cannot of course be excluded.

Our findings do indicate, therefore, that the deleterious effects of fluoride can be cancelled by the addition of calcium or magnesium salts. Evidence is available that the mortality produced in rats by an intake of sodium fluoride can be reduced by the introduction of calcium or magnesium salts.[25, 27, 28] On a similar basis calcium and magnesium salts have been used in the treatment of external fluoride burns.[12, 23] The importance of the studies reported in this paper lies in the demonstration that similar inactivation of fluoride can be produced in biological fluids. These findings also suggest that in systemic poisoning with fluoride an additional advantage may be derived from the use of calcium and magnesium salts. In clinical instances of acute fluoride intoxication hypocalcemic tetany has been observed.[1, 21, 24, 26] Similar deficits of magnesium have not been reported though they may well be present. Under these circumstances the administration of these salts will serve not only to cancel the fluoride effects, but will also replace any deficits of calcium or magnesium which may have developed. The need for prompt use of calcium or magnesium cannot be overemphasized. In the animal studies cited earlier the efficacy of this therapy in fluoride poisoning was markedly reduced after a five-minute delay and was virtually non-evident after ten minutes. *In vitro* studies of glucose metabolism in fluoride-treated blood indicate that if the introduction of the calcium or magnesium salt is delayed several hours the fluoride effect becomes, in some but not all instances, irreversible.[8]

Summary

1. The inhibition of glucose catabolism in defibrinated blood which follows the addition of sodium fluoride can be cancelled in part or entirely by the introduction of soluble salts of calcium or magnesium.

2. The significance of these findings in the therapy of fluoride poisoning has been discussed.

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THE METABOLISM OF POTASSIUM IN DIABETIC ACIDOSIS*

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Introduction

Although it has been known for some time that a loss of potassium from the body occurs in fasting[3, 18] and certain types of dehydration,[5, 13] and recently that the replacement of this potassium during parenteral therapy of the dehydration due to infantile diarrhea may materially reduce the mortality rate,[9] many of the changes that occur in the metabolism of potassium during the state of extreme dehydration associated with diabetic acidosis and coma have remained obscure. Potassium is lost from the body during the development of diabetic acidosis,[1, 6] and low serum levels of potassium have been reported during recovery.[7, 19, 23, 25, 28] Occasionally there have been associated symptoms attributed to these low levels.[23, 28] A study of potassium metabolism in diabetic acidosis was undertaken in the hope of defining more clearly the changes in potassium which occur during recovery from severe diabetic acidosis and, if possible, to ascertain whether it is necessary to replace potassium in these patients.

Experimental procedures and methods

The subjects studied were adult patients admitted in severe diabetic acidosis to the New Haven Hospital. In the first part of the study, the sera from blood samples drawn before institution of therapy were analyzed for sodium and potassium. Additional blood samples were drawn at four-hour intervals for the determination of sodium and potassium during the early phases of therapy, and at somewhat longer intervals thereafter.

In the second part of the study, the electrolyte balances of two patients were measured. Urines of these patients were analyzed for sodium, potasium, chloride, and nitrogen. In addition, sera were analyzed at the beginning and end of each period for sodium, potassium, chloride, and water. In one patient the balance of phosphorus was also measured.

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Because of the low serum potassium found in the initial stages of the study, it was thought desirable to administer potassium to these patients to ascertain whether any beneficial effects resulted. As low serum inorganic phosphate levels have been reported during recovery from diabetic acidosis,[19] it was felt that a solution of potassium phosphate should, on theoretical grounds, be best suited for this condition. Accordingly, varying amounts of a mixture of K_2HPO_4 and KH_2PO_4 of pH 7.4 were injected intravenously. Balances were measured to determine how much of the administered potassium was retained.

Electrocardiograms were also taken on these patients before, during, and immediately after each administration of potassium phosphate solution.

Sodium and potassium in serum and urine were determined by means of the flame photometer.[2] The techniques employed were those described by Hald.[20] Serum chloride was measured by Hald's modification of the Patterson micro method,[29] serum inorganic phosphate by the method of Fiske and Subbarow,[16] and serum bicarbonate content by means of the Van Slyke constant volume apparatus.[29] Serum water was estimated from the difference between wet and dry weights of one cc. aliquots. Blood sugar values were determined on Somogyi[31] filtrates according to the method of Benedict[4] using Rothberg and Evans tubes.[30] Hematocrits were measured by the use of Daland calibrated tubes.[12] Urine chlorides were determined by the Volhard-Harvey titration,[21] urine total nitrogen by the macro-Kjeldahl method,[29] and urine phosphate by the method of Fiske and Subbarow.[16]

Calculations

The calculations used in the balance studies were those devised by Elkinton which have been reported in detail.[13] Changes of extracellular fluid volume were calculated from the balance of chloride and the change in concentration of chloride in the serum. In this calculation it was assumed that after the patient had recovered from his acidosis and was adequately hydrated, the final extracellular fluid volume was equal to 20% of the body weight. Using this value and the chloride balance the changes in the chloride space could be extrapolated backward in time to yield a value for the initial extracellular fluid volume.

The changes in intracellular electrolytes were taken to be the difference between the total balance of each electrolyte and the amount which was calculated to be associated with changes of extracellular fluid volume. The concentrations of sodium and chloride in the extracellular fluid were calculated from their concentrations in the serum water using a Gibbs-Donnan

factor of 0.95. In the calculation of the concentration of potassium and phosphorus in the extracellular fluid a Gibbs-Donnan factor was not used as the amounts of potassium and phosphorus in the serum that are diffusible have not been clearly defined. Because of the relatively low concentrations of these ions in the serum, such a correction would not materially affect the final results.

In the processes of cell catabolism and anabolism, potassium is respectively lost and retained, since it is a normal constituent of cellular protoplasm. Using the nitrogen balance as an index of the final equilibrium between anabolism and catabolism an attempt was made to calculate the changes in potassium in "excess" of the changes in nitrogen. For this purpose a K : N ratio of 2.38 was used. This figure is taken from the data of Hastings[22] and is the lowest ratio found in analyses of the skeletal muscle of dogs by several observers.[10, 11, 15, 22, 26, 27] The highest ratio, 2.94, is that of Eichelberger.[11] The use of the highest K : N ratio would give a slightly lower value for the balance of the "excess" potassium in patient C.R., but would have a negligible effect on the total balance of "excess" potassium in patient F.O'K. as her overall nitrogen balance was only minus 1.7 gm.

Results

The chemical findings in the patients on whom serum studies were done are presented in Table 1. The changes in the serum electrolytes are correlated with the amount of fluid, glucose, sodium chloride, and insulin given, and with the output of urine. Most of these patients were in moderately severe acidosis as can be seen from their initial serum CO_2 contents, and several were in a moderate degree of circulatory collapse as evidenced by their initial blood pressures. However, with one exception, all patients responded well and made uneventful recoveries. The one exception, F.R., Table 1, had a severe degree of renal insufficiency complicating her diabetes. Although she recovered from her diabetic acidosis, her renal insufficiency gradually became more pronounced and she died two weeks after admission.

The most striking and consistent electrolyte changes were those in the concentration of serum potassium, which in all of these patients was initially elevated; but, with the inception of therapy, it diminished, and in one patient fell as low as 2.7 mEq. per liter. The changes in the serum potassium of all of the patients studied during recovery from diabetic acidosis are shown in Figure 1. The lowest concentrations occurred twelve to twenty-four hours after therapy had begun. At this point the serum potassium began to rise gradually, but usually required several days to reach the

TABLE 1

SERUM ELECTOLYTES AND TREATMENT OF SEVEN PATIENTS WITH DIABETIC ACIDOSIS

		Serum					*Blood*		*Cumulative Intake*				*Urine*
Patient	*Hours from start*	*Na mEq./liter*	*K mEq./liter*	*HCO_3 mEq./liter*	*Cl mEq./liter*	*Inorg. P mM./liter*	*Sugar mg.%*	*NPN mg.%*	*Water cc.*	*NaCl gms.*	*Glucose gms.*	*Insulin units*	*Volume cc.*
F.R.	0	131.0	8.0	2.8	85.4		1000						
B26145	16	133.9	3.9	14.0	94.3		800	114	4500	27.0	150	340	
	22	138.6	3.4				515		7500	40.5	225		
	64	132.6	4.7	10.2	95.9		600	118					
	88	127.5	4.8	8.5	91.8		490	170					
	112	130.0	4.2	13.7	83.2		429	177					
F.T.	0	142.0	7.5				600						
A77358	4	149.0	5.0						4200	31.5	120	160	
	8	143.0	4.4										
	10	144.0	4.6				95		6100	40.00	265	180	
	18	143.0	4.3									180	
	25	144.0	4.4										
	67			25.3	94.9		163	24					
D.H.	0	138.0	5.0	4.8	102.7		645						
B32297	4	145.0	4.0				470		3400	28.2	30	180	1090
	8	146.5	3.3				312		6700	47.7	89	280	1470
	12	146.0	3.0				340		7500	47.7	169	370	1900
	17	142.0	2.8				490		9000	54.0	240	440	2350
	22.5	138.0	2.7	15.2	123.1		146		11100	65.7	320	530	2443
	33	140.0	2.7				134		13400	72.0	490	560	3608
	46.5	141.0	3.9	18.1	122.6		310						6158

M.A.	0	134.0	5.6	7.2	92.0		736	26					
B47533	4	136.0	4.2				672		3465	30.6	90	150	950
	8.5	138.0	3.4				331		6165	46.8	180	240	1350
	12.5	145.0	3.3				174		7090	51.3	223	275	1550
	21	143.8	3.4	16.9	113.2		49	35	8215	54.0	306	290	1925
	25	141.0	3.7				93		8935	56.9	353	290	2325
	54.5	138.1	3.5	16.0	108		364		12090				3650
	80.5	138.5	3.6						15490				7750
T.H.	0	134.0	6.5				450						
A21257	4	138.0	4.5	21.4	102.6		328	53	2885	22.6	36	70	1030
	8.5	138.5	4.1				294		4840	34.1	104	70	1655
	13.5		3.9				240		6015	39.7	159	95	2135
	17		3.5				340		6565	39.7	214	125	2295
	25	138.0	3.8	17.1	113.8		375		6565	39.7	214	125	2795
C.R.	0	130.0	5.9	5.1	79.6	4.5	1472						
C4132	3.5	139.0	3.3			2.8	720					140	
	6.5	144.0	2.8		96.5	0.3	395		5870		110	160	750
	9.5	144.6	2.9			0.9	248		8250	36.4	162		
	13.5	143.9	3.2	21.9	103.4	1.2	126	53	10950		258	175	
	20	136.3	3.5		91.0	0.9	196		11920	63.4	360	200	1635
	28.5	141.7	4.2		95.2		148		13520		425		
	37.5	142.1	4.7	25.9	97.2		444	37	13770	67.0	458	225	3520
	61.5	139.3	4.5		98.0		380	27	15840				7180
F.O'K.	0	148.5	5.0	4.2	112.1	2.4	782						
C7548	5	152.5	3.3	9.8	126.4	0.4	384		4400	37.8		210	962
	15.5	147.3	2.5	16.4	116.7	0.3	288		7800	40.5	245	270	2612
	28	145.2	3.2		111.4	1.2	55	22	10575	45.8	305	305	
	39	143.0	2.9	20.0	108.0	0.8	281	23	11175	45.8	365	325	4142
	63	145.9	4.2	23.0	103.0	0.7	286	20	14175	49.3			
	87	149.4	4.8		112.0	1.2	252	25	15675	53.0			

normal level. These patients were observed carefully for any signs or symptoms which might result from a low serum potassium concentration, but none developed conspicuous muscular weakness or respiratory difficulty.

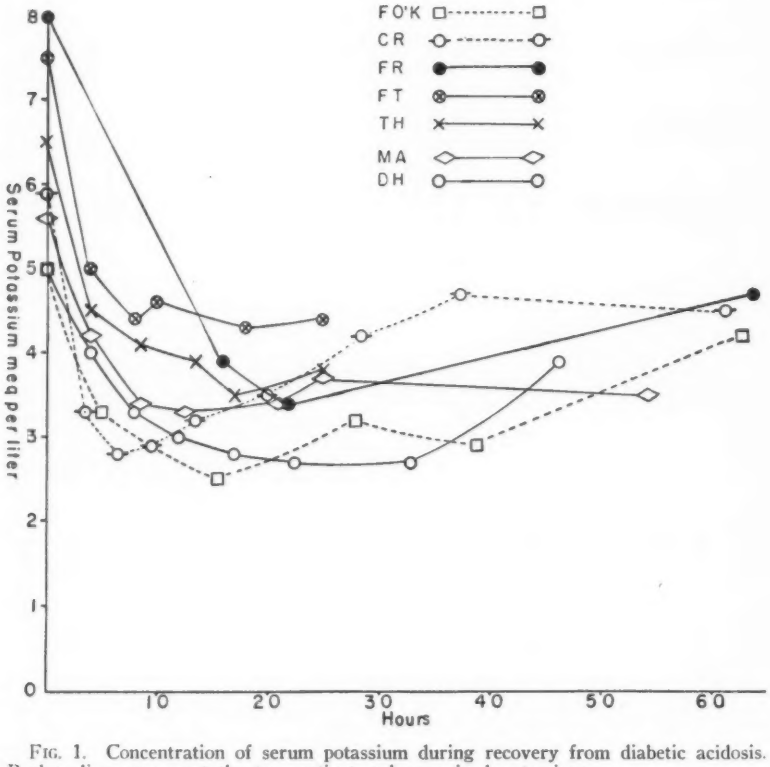

FIG. 1. Concentration of serum potassium during recovery from diabetic acidosis. Broken lines represent the two patients who received potassium.

Initial concentrations of sodium in the sera tended to be low, but rose quickly with therapy, and maintained a relatively constant level despite the parenteral administration of large amounts of salt and water. There was no consistent relation between the concentration of sodium and that of potassium in the serum. There was no obvious correlation between the level of blood sugar, which ranged initially from 450 to over 1,000 mg.%, and the severity of the acidosis or the level of the serum potassium.

The second part of the results deals with the data obtained from balance studies carried out on two patients during recovery from severe diabetic acidosis. In the first patient, C.R., sodium, potassium, chloride, and nitrogen balances were measured, and in the second patient, F.O'K., the phosphorus balance as well.

The changes in the blood chemistry, correlated with the therapy, are presented in Table 1. The intake, output, and balances of electrolytes are shown in Table 2, and the data derived by calculation in Table 3.

On admission both of these patients were comatose and had pronounced overbreathing. They were in severe diabetic acidosis with initial CO_2 levels of 5.1 and 4.2 mEq. The initial serum sodium of C.R. was low, but that of F.O'K. was considerably higher than normal, and remained so after her recovery from acidosis.

The initial serum potassium concentrations, like those of the preceding five patients, were elevated, and fell rapidly during the early hours of therapy. The lowest concentration observed, 2.5 mEq., occurred in F.O'K. fifteen hours after initiation of treatment, but was not accompanied by symptoms or signs of hypokalemia.

The changes in the serum inorganic phosphorus were even more striking than those of the serum potassium. Like that of potassium the initial concentration of phosphorus tended to be elevated and to fall with therapy, until low levels were reached. However, the fall in serum phosphorus was much more abrupt, suggesting that the early changes in serum potassium and phosphorus are probably coincidental rather than related.

A mixture of the monobasic and dibasic salts of potassium phosphate was administered to the two patients, C.R. and F.O'K. In the first patient, C.R., the initial dose of potassium phosphate containing 54 mM. of potassium and 30.2 of phosphorus, dissolved in 1,000 cc. of 5% glucose, was given intravenously over a period of four hours. It was begun ten hours after initiation of therapy. The serum potassium immediately before the administration of potassium of 2.9 mEq., and shortly after the completion of the infusion was 3.2 mEq. The T waves in an electrocardiogram before potassium was given were inverted or isoelectric; after its administration they were more upright.

A second dose of potassium phosphate, containing 81 mM. of potassium and 45.3 of phosphorus, again dissolved in 1,000 cc. of 5% glucose, was given over a period of four hours, beginning twenty hours after therapy was started. A serum potassium immediately before was 3.5 mEq., and one hour after administration of potassium was 4.2 mEq. The T waves in the electrocardiogram were now upright in all leads. This was the only objec-

TABLE 2

EXTERNAL EXCHANGES OF ELECTROLYTES AND NITROGEN

	Time		Intake*					Urine						Balance				
Patient	Hours from start	Period	Cl mEq.	Na mEq.	K mEq.	P mM.	N gms.	Vol. cc.	Cl mEq.	Na mEq.	K mEq.	P mM.	N gms.	Cl† mEq.	Na† mEq.	K mEq.	P mM.	N gms.
C.R.	8	I	680	725	2		8.3	750	2	20	14		2.9	+619	+645	—16		+ 5.4
C4132	21	II	462	462	54		0	885	22	19	25		8.6	+433	+434	+29		— 8.6
	38	III	62	62	81		0	1885	105	71	53		15.6	— 97	— 64	+24		—15.6
	62	IV	40	46	35		5.0	3660	423	403	74		23.1	—385	—360	—39		—18.1
Total	62		1244	1295	172		13.3	7180	552	513	166		50.2	+570‡	+655‡	— 2‡		—36.9‡
F.O'K.	5	I	651	678	0	0	8	962	168	86	13	1.2	2.4	+480	+589	—13	— 1.2	+ 5.6
C7548	15.5	II	46	46	0	0	0	1650	241	90	24	1.1	3.9	—196	— 46	—24	— 1.1	— 3.9
	39	III	80	85	120	62.0	1	1530	173	10	27	18.4	3.8	— 99	+ 67	+93	+43.6	— 2.8
	63	IV§	60	75	162	66.9	4.1	4000	367	180	63	41.2	4.7	—311	—108	+99	+25.7	— 0.6
	87	V	68	62	134	5.4	5.0	1200	198	116	56	14.1	5.1	—133	— 58	+77	— 8.7	— 0.1
Total	87		905	946	416	134.3	18.1	9342	1147	482	183	76.0	19.9	—259	+444	+232	+58.3	— 1.8

* Both oral and parenteral.
† Corrected for small amount of Cl and Na in blood taken for analysis.
‡ Corrected for approximately 1 liter of vomitus lost during the first three periods.
§ A small amount of liquid stool was not collected during this period.

TABLE 3

EXCHANGES OF ELECTROLYTES BETWEEN EXTRACELLULAR AND INTRACELLULAR PHASES OF BODY FLUIDS

	Time			*Changes in Amounts of*						
				Na		*K*			*P*	
Patient	*Hours from start*	*Period*	*Extracellular fluid volume liters*	*Extracellular mEq.*	*Intracellular mEq.*	*Extracellular mEq.*	*Intracellular mEq.*	*In excess* mEq.*	*Extracellular mM.*	*Intracellular mM.*
C.R.	0	0	7.8							
C4132	8	I	12.0	+730	— 87	—12	— 4	—17		
	21	II	17.2	+605	—168	+26	+ 3	+24		
	38	III	15.2	—200	+139	+12	+12	+49		
	62	IV	11.6	—551	+194	—20	—19	+24		
Total	62			+584	+ 78	+ 6	— 8	+80		
F.O'K.	0	0	17.3							
C7548	5	I	18.8	+280	+308	—25	+12	— 2	—35.0	+33.8
	15.5	II	19.2	—100	+ 54	—14	—10	— 1	— 1.6	+ .5
	39	III	20.1	+ 30	+ 37	+10	+82	+88	+11.4	+32.3
	63	IV	18.4	—199	+ 91	+19	+80	+81	— 4.3	+30.0
	87	V	16.1	—329	+271	0	+77	+77	+ 7.0	—15.7
Total	87			—318	+761	—10	+241	+243	—22.5	+80.9

* Intracellular K in excess of nitrogen.

tive evidence of any beneficial effect. There was no dramatic change in the patient's clinical condition.

The second patient, F.O'K., received slightly larger doses of potassium phosphate seventeen hours after therapy began, at a time when her serum potassium was 2.5 mEq., and her serum phosphorus 0.3 mM. per liter. Her first dose contained 109 mM. of potassium and 60.4 of phosphorus, and was given intravenously in 1,500 cc. of 5% glucose. Because of technical difficulties with the infusion set, this required about eight hours. Two hours after the infusion finished, a serum potassium was 3.2 mEq. per liter and a serum phosphorus was 1.2 mM. per liter. During this period the patient also received 11 mM. of potassium and 1.5 of phosphorus by mouth.

The second injection of potassium phosphate, begun forty-one hours after admission, contained 109 mM. of potassium and 60.4 of phosphorus in 1,500 cc. of 5% glucose which was given over a four-hour period. The serum of blood drawn eighteen hours later contained 4.2 mEq. of potassium and 0.7 mM. of phosphorus per liter. During this period the patient also received 53 mM. of potassium and 6.4 of phosphorus by mouth. In the last period the patient was given 134 mM. of potassium and 5.3 of phosphorus by mouth. Serum potassium at the end of this period was 4.8 mEq., serum phosphorus was 1.2 mM. per liter.

In Tables 2 and 3 the changes in intracellular and extracellular electrolytes are shown. At the time when the serum potassium of these patients was lowest, no potassium had been taken up by the cells. Actually in patient C.R. an additional amount seems to have been lost from the cells. Only when exogenous potassium was supplied to the body did the cells take up potassium in excess of nitrogen. In contrast to the changes in potassium, in F.O'K. it can be seen that almost all of the phosphorus which left the extracellular fluid during the first few hours of therapy was taken up by the cells.

In Table 3 are also shown the changes in intracellular sodium. In C.R. the amounts of sodium either going into or leaving the cells during the various periods studied are small and probably insignificant. In F.O'K., however, during each period the cells seem to have taken up varying amounts of sodium, so that at the conclusion of the study over 700 mEq. seem to have gone into some part of the intracellular fluid.

Discussion

Although patients in diabetic acidosis appear to have varying degrees of potassium deficiency, [1, 5] the initial concentrations of serum potassium in

these patients were elevated. In the two patients in which it was estimated, however, the total amount of potassium in the extracellular fluid was not increased. The initial elevation of the concentration of serum potassium, therefore, seems to be due to contraction of the extracellular fluid volume rather than an actual increase in the amount of extracellular potassium. Why the concentration is not kept at a more normal level is not clear, but this may be due to decreased kidney function secondary to circulatory collapse.

The fall in the serum potassium concentration which has been observed in these patients, and in other patients recovering from diabetic acidosis,[7, 19, 23, 25, 28] may theoretically be due to four factors: (i) Expansion of the extracellular volume resulting from the administration of large quantities of fluid; (ii) excretion of potassium by the kidney; (iii) transfer of potassium to the red cells; (iv) transfer of potassium to tissue cells.

Expansion of the extracellular fluid undoubtedly plays a significant part in causing the serum potassium concentration to decrease. The volume of extracellular fluid of these patients is greatly contracted at the height of their acidosis; with therapy it is rapidly re-expanded, and often temporarily over-expanded. This expansion of extracellular fluid volume will by simple dilution cause the concentration of serum potassium to diminish. In the first patient, C.R., at the time when the serum potassium concentration was lowest, the extracellular fluid volume had risen from 7.8 to 12 liters, an increase of approximately 50% over its original volume. It is obvious that in this patient, the early decrease in serum potassium concentration was in a large measure due to dilution as a result of the expansion of the extracellular fluid volume. However, in F.O'K. expansion of extracellular fluid seems to have been of less significance as a cause of the decrease in serum potassium. Fifteen and a half hours after the beginning of therapy her serum potassium reached its lowest concentration of 2.5 mEq. per liter, a percentage drop from its original concentration of the same magnitude as that of patient C.R. Nevertheless, in this patient the extracellular fluid had increased only from 17.3 to 19.2 liters, or 11% as compared to the 50% increase of extracellular fluid of C.R.

In view of the relatively small increase in the extracellular fluid volume of this patient, at the time when her serum potassium was lowest, other factors besides dilution must have been responsible for the decrease. In Atchley's study the reinstitution of insulin therapy in the one patient who developed acidosis diminished sharply the urinary excretion of potassium. In the first three days of recovery this patient excreted only 10.3 mEq. of

potassium in the urine. This observation has tended to minimize the importance of excretion of potassium by the kidneys as a cause of the decrease in serum potassium concentration. However, in patients C.R. and F.O'K., excretion of potassium by the kidneys was not as sharply curtailed. In patient C.R., during the first eight hours of therapy, 14 mEq. of potassium were excreted in the urine. As can be seen in Table 3, most of this was derived from the extracellular fluid. This loss through the kidneys is equivalent to about one-fourth of the amount originally present in the extracellular fluid, and in the absence of replacement either from an exogenous source or by a further release of potassium from the cells would contribute significantly toward the observed decrease in serum potassium concentration. In F.O'K. during the first fifteen and a half hours after the beginning of therapy, and at the time her serum potassium was lowest, 37 mEq. of potassium had been excreted by the kidneys. This is approximately 40% of the total originally in her extracellular fluid, and seemed to be the most important factor in causing the decrease in serum potassium concentration. It is interesting to note that at a time when the serum potassium concentration of these patients was decreasing to low levels, the kidneys continued to excrete urine containing 15 to 19 mEq. of potassium per liter. This observation would seem to indicate that the kidneys do not conserve potassium even when there is a deficiency of this substance in the body. This observation is in agreement with that of Elkinton and Tarail[32] who found that the kidneys of patients with potassium deficiencies and low serum potassium concentrations did not seem to conserve potassium.

The third possible factor, movement of potassium from the serum into the red cells, is believed to contribute little to the decline of serum potassium. It is known that red cells are depleted of their potassium content during the development of acidosis[7, 19]; but Danowski, Hald, and Peters[7] found that these cells did not begin to take up potassium until after the serum potassium had reached its lowest concentration. In some of their cases the red cells actually continued to lose potassium during the early phases of recovery, while the serum potassium was decreasing. In F.O'K., no potassium had been taken up by the red cells during the first twenty-four hours.[24]

The fourth factor is the movement of potassium from the extracellular fluid into other cells of the body. According to the studies of Atchley *et al.*[1] and Butler,[6] during the development of diabetic acidosis large amounts of potassium in excess of nitrogen are lost from the body. The only possible source of such large quantities is the cells. Hence, one can say that in severe

diabetic acidosis cells of some, if not all, tissues are depleted of potassium. That the red cells are depleted has been demonstrated by direct analysis.[7, 19] When treatment for acidosis is begun, the processes which resulted in the loss of cellular potassium are reversed and potassium is taken up by the cells in excess of nitrogen. However, in Table 3 it can be seen that the initial fall of serum potassium is not due to a movement of potassium into the cells. In C.R. and F.O'K., at the time when the serum concentration of potassium was lowest, no potassium had been taken up by the cells. In fact, in C.R. the cells seem to have released an additional small quantity of potassium in excess of nitrogen. As soon as exogenous potassium was supplied to these patients, however, a considerable amount of potassium was taken up by the cells in excess of nitrogen. This occurred in both cases during the three periods in which potassium was given, and at the conclusion of the study the cells were still retaining potassium in excess of nitrogen, so that the exact amount which might eventually have gone into the cells can not be accurately stated. This would seem to agree with observations of Elkinton, Winkler, and Danowski,[14] who, in their studies of the effect of various alterations of body water and electrolytes, found that appreciable amounts of potassium were transferred into cells only when exogenous potassium was available.

The movements of potassium in these patients may be interpreted in the following manner. During the development of severe diabetic acidosis, presumably as a result of starvation and dehydration, potassium in excess of nitrogen is lost from the cells. When insulin therapy is resumed this loss is abruptly halted. At this time the total amount of potassium in the extracellular fluid is normal or slightly increased, but as a result of the diminution of the total volume of the extracellular fluid from dehydration its concentration is increased. When therapy is begun the loss of potassium from the cells in excess of nitrogen ceases, and the extracellular fluid is deprived of its cellular source of potassium. As a consequence, when the extracellular fluid volume is re-expanded, and a portion of the extracellular potassium is excreted by the kidneys, the concentration of potassium in the extracellular fluid falls to the low levels which have been observed. Why none of the extracellular potassium moves into the cells immediately is not clear. If transfer of potassium into the cells is dependent upon an exogenous supply,[14] the earlier administration of potassium solutions to these patients might not only prevent such precipitous drops in serum potassium but allow earlier replacement of the cellular deficit of potassium.

The movements of inorganic phosphorus in the one patient studied seem

to be conditioned by other factors than the ones affecting potassium. Like potassium the concentration of serum inorganic phosphorus tends to be high initially and to fall abruptly with therapy. However, of the 34.7 mM. of phosphorus which were calculated to have left the extracellular fluid during the first five hours of therapy, 33.5 mM. must have gone into the cells, as only 1.2 mM. of phosphorus were excreted during this time. Thus, the early fall of serum inorganic phosphorus is not due solely to dilution or to excretion, but seems to result from the movement of phosphorus into the cells. This movement may be made in response to reactions which are initiated in the cells as a result of the administration of insulin.

The movements of sodium into and out of the cells in these two patients followed no definite pattern. In C.R. the movements of sodium were not consistent, and the quantities were small. In view of the inherent error involved in calculating the movements of intracellular sodium the changes in this case are of no significance. In F.O'K. the changes are greater in magnitude and consistent in direction. Throughout all periods sodium seems to have left the extracellular fluid, so that at the conclusion of the study, a total of approximately 761 mEq. appears to have moved into some phase of intracellular fluid. Muscle cells comprise a large percentage of the total body cells. Since normally they contain only small amounts of sodium, it is unlikely that they took up such a relatively large amount of sodium. It is possible that most of this sodium may have gone into some repository such as bone which contains appreciable amounts of sodium. Another possible explanation is that certain cells of the body which normally contain large amounts of sodium took up most of the 761 mEq.

The findings in these patients and in those reported by others[6, 7, 23, 25, 28, 17] make it advisable to review the use of solutions containing only sodium chloride and glucose, and to consider the replacement of potassium and phosphate. It seems well established now that these patients develop varying degrees of potassium and phosphate deficiency during the development of acidosis, and that in the majority the serum potassium and inorganic phosphate decrease to low levels during therapy. Whether this decreased concentration of potassium and phosphate in the serum in addition to the total body deficit is harmful to the patients is difficult to evaluate. In a few of the cases such as the one described by Holler[23] there are clear indications that the administration of potassium was of benefit. Such objective evidence of potassium deficiency as the occurrence of muscular paralysis must be infrequent, but the possibility of deleterious effects which may be more subtle in their manifestations must be considered.

In view of the potassium and phosphate deficits, it would seem advisable to test the therapeutic efficacy of both potassium and phosphate solutions in the treatment of diabetic acidosis. After the administration of potassium phosphate there was no obvious clinical improvement in these patients, although in the electrocardiogram the T waves, which had previously been inverted or isoelectric, became more upright. After about one-third of the initial dose of potassium phosphate had been given to F.O'K. her mental state seemed much improved. Since she was well on the way to recovery before receiving the potassium phosphate, this response is difficult to evaluate. In the study of Franks[17] it is interesting that the most consistent change noted when sodium phosphate was given to sixteen patients with diabetic acidosis was a rapid improvement in their mental states. As the initial serum potassium of these patients is high, and their renal function, owing to circulatory collapse, is often poor, the administration of potassium is not without danger. If it is given too early or too rapidly, it may cause potassium intoxication. It would seem advisable to wait after initiation of normal saline and glucose therapy until the serum concentration of potassium has begun to fall and excretion of urine has been restored before giving potassium.

The amount of potassium which these patients should receive has not yet been clearly defined. Patient C.R. retained 80 mEq. of potassium in excess of nitrogen, or approximately 1.5 mEq. per kilogram, while F.O'K. retained 243 mEq., or 4 mEq. per kilogram without developing hyperkalemia. In the group of patients studied by Danowski *et al.*,[8] larger amounts of potassium per kilogram seemed to be retained, one patient retaining 8 mEq. of potassium per kilogram, but the concentrations of potassium in the serum sometimes rose above normal limits.

Whether the administration of potassium to patients in diabetic coma will reduce the mortality or morbidity remains to be seen. A more prolonged trial of replacement therapy will be necessary to evaluate any beneficial results.

Summary and conclusions

1. The concentrations of sodium and potassium in the sera of five patients with diabetic acidosis were measured at short intervals during recovery. In two additional patients balances of sodium, potassium, chloride, and nitrogen were measured as well. In one of these two the balance of phosphorus was also measured.

2. The serum potassiums of all these patients were elevated on admission, but during the early hours of therapy fell sharply, in all but two patients

reaching a concentration of less than 3.5 mEq. per liter.

3. The initial elevation of serum potassium concentration seems to be due to hemoconcentration and possibly to failure of excretion of potassium by the kidneys owing to circulatory collapse.

4. In two patients in which it was studied the serum inorganic phosphate concentration was high initially and fell precipitously with therapy.

5. Potassium phosphate solutions were administered to these two patients and the amounts of potassium and phosphorus taken up by the cells were calculated.

6. The decline in serum potassium seems to be mainly a result of expansion of extracellular fluid and excretion of potassium by the kidneys, which continues even when the concentration of potassium in the serum is low. The fall in serum phosphate seems to be due primarily to movement into the cells. Potassium was not taken up by the cells until an exogenous supply was made available.

7. The significance of these findings and their possible therapeutic implications have been discussed.

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INCIDENTAL FINDING OF MEGALOBLASTIC-LIKE CELLS IN BONE MARROW OF ONE OF TWO SWINE WITH MACROCYTIC ANEMIA AND ACHLORHYDRIA*†

F. DOUGLAS LAWRASON‡ AND E. P. CRONKITE

Introduction

Ever since Ehrlich described the megaloblasts seen in pernicious anemia during relapse, there have been attempts by many investigators to produce experimentally an anemia similar in all respects to human pernicious anemia. Macrocytic anemia with the megaloblastic type of erythrocytic regeneration in the bone marrow, achlorhydria, and a specific response to a substance containing the anti-anemic principle are the criteria necessary to establish the presence of such an anemia.

Thus far, efforts to produce a macrocytic anemia in experimental animals have not been uniformly successful, but it is agreed by many investigators that swine constitute a suitable experimental animal in which to study this problem.[3, 16, 25, 32] Total gastrectomy[1, 9, 15, 17, 18, 30] alone, or combined with liver damage,[23] has produced only an occasional macrocytic anemia in animals. The usual resultant anemia is normocytic and hypochromic and in most cases responds to iron therapy. Davis[4] reported the development of a "hyperchromic" anemia in dogs given daily doses of choline chloride. This not only responded to liver therapy but also to atropine sulfate, and on this basis the anemia was not considered to be due to a specific lack of the anti-anemia factor.

Of extreme interest are the recent reports of Welch,[25] Heinle,[10] Cartwright,[2] and their associates, who have been able to produce macrocytic anemias in swine fed diets deficient in pteroylglutamic acid and supplemented with a chemical antagonist of pteroylglutamic acid. The difference

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* The opinions or assertions contained in this article are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the Naval service at large.

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in the basic diets fed to the different groups of swine by the above investigators may be relevant in attempting to explain the varying response of the experimental anemias in these animals to treatment with extrinsic factor and normal human gastric juice, liver extract, and an increase in protein in the diet. However, the anemia of all the animals rendered deficient in pteroylglutamic acid did respond favorably to treatment with pteroylglutamic acid. Experimental macrocytic anemias can also be produced by feeding diets deficient in B complex vitamins but this method is not entirely satisfactory.[3, 16, 24, 25, 26, 30] Many of the anemias produced by these means were found to respond to a factor contained in yeast, related possibly to the vitamin B complex group, yet not identifiable with any one vitamin, and at times unresponsive to purified liver extracts.[17, 19, 27, 28, 32] In view of these experimental data and clinical evidence, Wintrobe[30] and others have raised the question whether or not pernicious anemia is in reality a multiple deficiency disorder rather than one caused by the lack of a single substance.

The identification of the megaloblast presents a cytological problem that has been the cause of much disagreement for many years. Jones,[11, 12, 13] Downey,[7, 8] Wintrobe,[31] and others believe that megaloblasts are present only in fetal bone marrow and in pernicious anemia or anemias in which the antipernicious anemia principle is lacking, and are not found in normal bone marrow. On the other hand, certain hematologists, principally Sabin[20, 21] and Doan,[5, 6] consider the megaloblast as an early stage in the development of the erythroblast and to be present in normal bone marrow. The main criticism of this latter observation is the technique of supravital staining upon which it is based. Jones contends that the delicate chromatin pattern of the nucleus, which is seen in the dry smear and lost in the supravital stain, is the most important criterion for the identification of the megaloblast. At the same time he finds it quite impossible to identify the megaloblast in imbedded tissue sections and would thus be unable to confirm their presence in the bone marrow of the experimental monkeys with macrocytic nutritional anemia as reported by Wills *et al.*,[26, 28] and in the swine with anemia as claimed by Miller and Rhoads.[16] Wintrobe[32] in 1939 reported the presence of cells which had the appearance of "megaloblasts" in the bone marrow of pigs with normocytic anemia due to a deficiency of water-soluble vitamins and other substances contained in yeast. He was unable to make a positive identification of those cells at that time. However, recently, two groups of investigators have reported the presence of cells resembling megaloblasts in the bone marrow of swine deficient in pteroylglutamic acid.[2, 25]

The following preliminary report is based on observations made in two swine during seventeen months following exposure to atom bomb ionizing radiation at Bikini. Both hogs were in the group of experimental animals placed aboard target ships to be studied for the effects of total body radiation from the air-burst bomb. Animal A received minimal amounts of ionizing radiation while animal B was exposed to considerable amounts of radiation.

Experimental background

In order to evaluate the hematologic changes found in the two swine during the seventeen months following exposure to ionizing radiation, it will be necessary to examine in some detail the background and the possible exciting causes which may have been involved in the development of the macrocytic anemia. Both animals were fifteen weeks of age at the time of exposure, 1 July 1946, and each had been observed for about six weeks prior to that time. They had been obtained from a single herd, predominantly of Duroc-Jersey stock, with an admixture of Chester-White and Poland-China stock. At that time agglutinations for Brucella were negative.* During the period aboard ship and continuing for eighty-five days following exposure, all swine were maintained on a diet somewhat restricted in calories. However, except for the restriction in total calories, the diet was considered nutritionally adequate for growing swine in respect to protein, vitamin, and mineral requirements. The swine gained an average of one pound a day in weight during the fifteen-day period before the detonation of the bomb. Animals A and B gained at this rate and continued to develop to the normal adult size weighing approximately 200 pounds each. The diet consisted of crushed oats 90%, meat scraps 10%, and a swine salt mixture 1%. Alfalfa hay was given *ad lib.* until the supply was exhausted, approximately ten days before the animals were transferred from the ship to the Naval Medical Research Institute, Bethesda, Maryland. During observation at the Institute, their diet was basically composed of 17% protein-mineral mixture and 83% whole yellow corn as recommended by the U.S. Department of Agriculture. A commercial vitamin-mineral mixture supplied the necessary vitamins and minerals. For the period of six months after arrival at the Research Institute, alfalfa meal was not available so the tankage was increased proportionately. The amount of the

* Agglutination tests were performed by the California State Department of Agriculture.

diet fed the animals once daily depended upon the amount consumed *ad lib.* with a portion being left in the feeding trough.

In addition to the above dietary changes, the following factors might also be considered as variables in the overall experiment involving swine at Bikini. Approximately ten days after the bomb burst the herd was found to be infected with *Ascaris suis*. Sodium fluoride, 1% by weight of a daily ration, was given once to each pig. This amounted to about 9 grams of sodium fluoride per animal, a dose considered sufficient to eradicate the infestation.

Another factor which may have contributed to a nutritional deficiency and may have altered the blood picture was chronic hog cholera. Aboard ship a few animals died; the clinical course and autopsy findings of these animals were similar to those of animals that have since died of proven atypical chronic hog cholera* at the Naval Medical Research Institute. These animals presented a profound picture of malnutrition. The incidence and the effect on hematopoiesis of chronic hog cholera in the herd aboard ship and after arrival at the Institute are unknown. Undoubtedly, a chronic infection such as chronic hog cholera would influence the hematologic profile of the affected swine. The influence of age on hematopoiesis is of importance and will be considered along with the "observations."

Observations

Figure 1 shows a comparison of the total RBC of the heavily irradiated swine, the biologically insignificantly irradiated swine aboard the target ships, the swine which were retained aboard the animal laboratory ship, the *USS Burleson,* and the two animals under consideration in this report. It will be noted that all control counts were between 5.5 and 6.0 million. Scarborough[22] reports that the normal range of the RBC in swine is between 5.0 and 9.0 million, with an average of 6.7 million. Likewise the control hemoglobin, hematocrit, and blood indexes were within the normal range. The RBC of both the heavily irradiated and insignificantly irradiated groups (*i.e.,* 90 animals aboard target ships that were beyond the range of significant radiation) decreased at about the same rate and followed the same trend. The RBC of the swine that never left the *Burleson* also were found to be as low as those of the test animals 30 days after the test. All three groups recovered at about the same rate, and the beginning of the recovery period coincided with the correction of the calorically restricted

* Proved by biologic test by the U.S. Department of Agriculture.

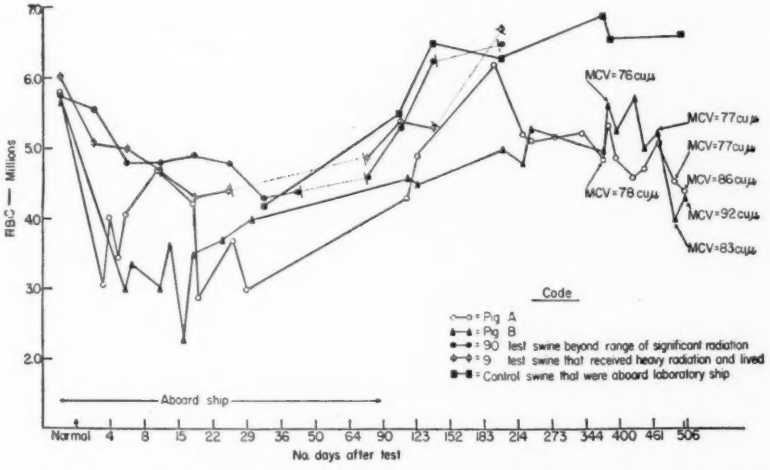

FIG. 1. Comparison of RBC of swine A and B with heavily irradiated and insignificantly irradiated swine exposed to atomic bomb air burst test.

diet. The final counts at the time of this study in the mature animals (608 days) ranged between 6.5 and 7.0 million RBC per cu. mm. Since Wintrobe,[29, 32] Smith,[24] and others have stressed the importance of nutrition, initial estrus, and other factors and their effect upon the hematologic status in young swine, it is realized that these factors probably played an important part in the overall blood picture and are possibly reflected in the trend of the RBC of the animals.

The course of the RBC in animals A and B can also be followed over this period of time. In regard to the RBC, however, neither of these animals completely recovered as the rest of the group did. The peripheral blood has continued to manifest moderate degrees of anisocytosis, poikilocytosis, and polychromasia, and in many respects these abnormalities have become more pronounced (Fig. 2). The volume of the red cells has been considerably greater than normal. The mean corpuscular volume (MCV) of animal A has been greater than 71 cubic microns in all determinations except one; the last determination at the time of this study was 86 cubic microns. Likewise, the MCV of pig B has become progressively greater; the last five determinations were 76, 74, 77, 83 and 92 cubic microns. The mean diameters of the RBC are increased as well in both animals. This is considered a true macrocytosis and not just a reflection of a reticulocytosis,[14]

since on repeated determinations the reticulocyte count has ranged between 0.5 and 1.3%.

Animal A received a minimal amount of ionizing radiation, probably in the range of 50 roentgens, calculated from the dosage recorded by film in the same compartment aboard the target ship and from the biological effect. This latter effect is studied best at the present time by following the total WBC* and differential counts after exposure to radiation. Figure 3 graphically compares the total WBC in the same two groups and animals A and B throughout the same period of time. In the immediate post-irradiation

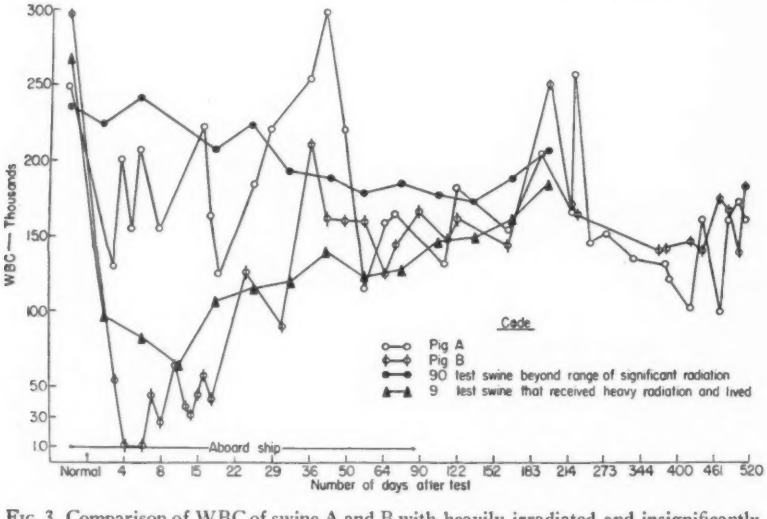

FIG. 3. Comparison of WBC of swine A and B with heavily irradiated and insignificantly irradiated swine exposed to atomic bomb air burst test.

period, pig A showed only a moderate drop, not of the same degree as the average leucocyte decrease of the nine more heavily irradiated animals that survived at least six months. The recovery of the WBC occurred gradually over three to five months.

The typical leucocyte response following irradiation of pigs is clearly outlined in the course of the WBC in animal B. Of the two pigs in the same

* Although the total lymphocyte count is generally considered as the best index to exposure to radiation, the statistical analysis of Mr. W. V. Charters (Statistical Division of the Bureau of Medicine and Surgery) has shown the total WBC of swine to be equally significant (unpublished).

compartment aboard the target ship with B, one was found dead on the first day and its death can not be satisfactorily explained. The second died on the fifth day with a generalized hemorrhagic diathesis. The photographic dosimeter in the compartment recorded about 3,500 roentgens of radiation. However, the fact that animal B is still alive constitutes evidence that it received only a fraction thereof. A possible explanation is that the animal may have been protected by heavy machinery or other shielding. The higher average WBC seen early in the experiment may have been due to immaturity of the entire group.

Repeated gastric analyses before and after histamine failed to reveal any free hydrochloric acid in either of these animals. However, Wintrobe[32] states that the achlorhydria found in his experimental swine was not persistent and varied with repeated determinations. Icteric index and Van den Bergh determinations have been within normal limits. Since it was not feasible to carry out serial bone marrow studies on all experimental animals, early bone marrow imprints are not available. Studies of the marrow were first performed on animals A and B approximately one year after exposure to the atom bomb and have been repeated at monthly intervals. The technique of repeated sternal aspiration and examination of the dry smear-imprint has been entirely satisfactory.

The bone marrow from animal A is moderately hyperplastic with a definite increase in the number of early erythrocytic cells. The myelo-erythrocytic ratio is 3 to 1. The majority of the immature erythroid elements manifest the usual pattern of the normoblastic series. Their nuclei are composed of relatively thick strands of chromatin which have the early tendency to clump, and as the cell matures, it assumes the "Radkern" type nucleus. These cells are of normal size and contain the normal nucleocytoplasmic ratio. However, there is a significant number of immature erythroid cells that markedly resemble the megaloblasts seen in the bone marrow of pernicious anemia in relapse (Fig. 4). The very early forms, the "pro-megaloblasts," are larger and contain more cytoplasm than the pro-normoblasts seen in the bone marrow of the control animals. The chromatin of the nucleus has a fine, reticulated pattern which is distributed uniformly throughout. The delicate strands are widely separated by parachromatin and fail to show any tendency to clump. One or two nucleoli are usually present. We have labeled the next distinct stage in maturation the "basophilic megaloblast"; these are comparable to the basophilic normoblast of normal erythropoiesis. These cells also are larger and contain more cytoplasm which is somewhat less densely basophilic than its counterpart

in the normoblastic series. The chromatin pattern of the nucleus continues to show the delicate scroll design with little evidence of clumping. About 30 to 40% of the immature hemoglobiniferous cells at this stage are of the megaloblastic-like type.

As maturation proceeds, the nuclear distinction between the two series becomes less marked since there is also evidence of clumping of the "megaloblastic" nucleus. However, these near-mature "megaloblasts" continue to be considerably larger and in many cases are twice the size of the corresponding polychromatic or orthochromatic normoblast. Furthermore, the cell contains more cytoplasm and the nucleus remains larger. That these cells are the precursors of the macrocytes seen in the peripheral blood is apparent through the various intermediary stages in their maturation. Only an occasional, large neutrophilic meta-myelocyte, like those which have been described in the megaloblastic marrow of pernicious anemia, is seen. Multilobulation of the nucleus of the mature neutrophil in the peripheral blood is absent.

In comparing the typical megaloblasts found in pernicious anemia with the megaloblastic-like cells in animal A, differences can be noted. However, these differences are believed by the authors to be for the most part interspecies differences, since in the marrow of swine, these cells differ as markedly in their characteristics from the usual normoblastic pattern seen in the controls as the human megaloblast does from its normoblastic counterpart. It is believed that all of the cytological criteria for the identification of the megaloblast are present.

Animal B also manifests a macrocytic anemia with anisocytosis, poikilocytosis, and polychromasia. This animal definitely received more radiation than pig A, but from the hematologic standpoint they have followed a similar course over the past eight months. As yet no cells similar to megaloblasts have been found in the bone marrow of animal B even though a moderate degree of erythroid hyperplasia is present. It has been considered desirable to observe the spontaneous course of these two animals over a longer period of time; therefore, specific therapy has been withheld.

Comments

It is not the aim of this report to propose a relationship of cause and effect between any of the variables mentioned and the appearance of the "megaloblastic" macrocytic anemia. There is little possibility of complete evaluation of the important variables of nutrition, radiation, and disease that were present during the development of these swine. However, it

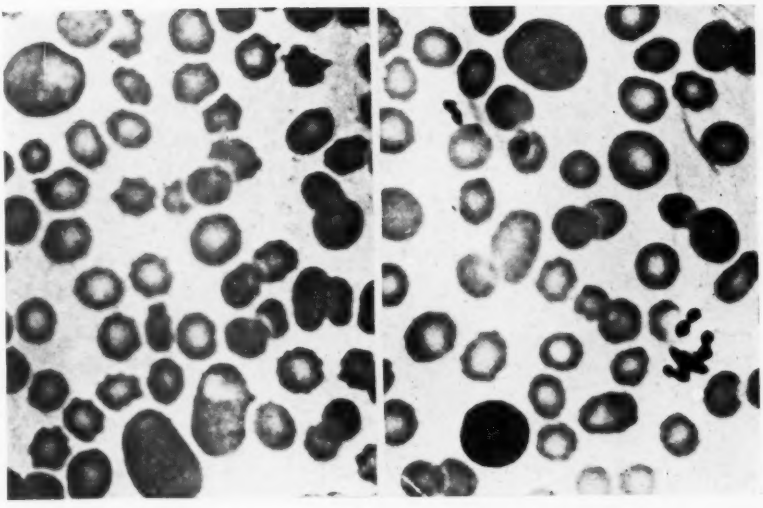

FIG. 2. Peripheral blood smears from pig A (left) and pig B (right).

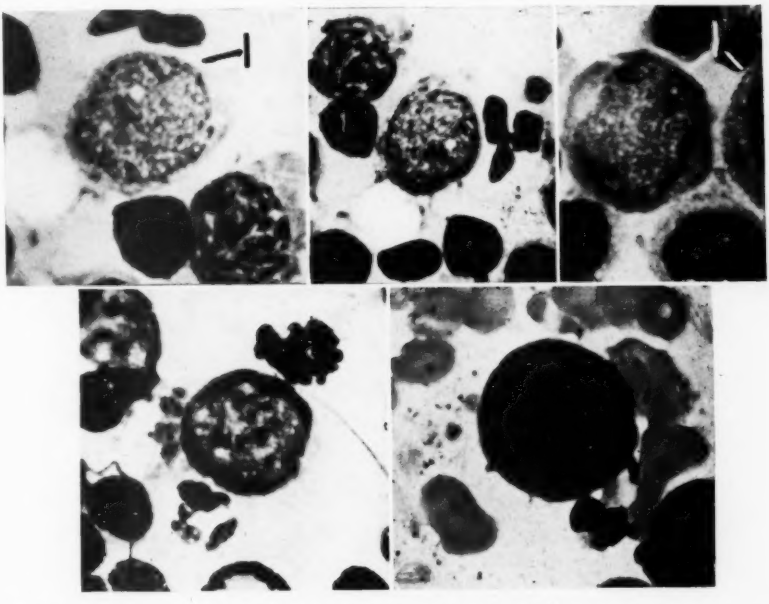

FIG. 4. Bone marrow from pig A showing immature erythrocytes which demonstrate the characteristics of the megaloblast.

would seem that a dietary factor probably played an important role in the development of the macrocytic anemia and associated bone marrow findings. It would be difficult to ascribe the hematologic findings to the direct effects of radiation. It may be that a combination of the two, plus possibly other undetermined factors, synergistically led to the development of such an anemia.

In any event, the finding of megaloblastic-like cells in the bone marrow of an animal manifesting a pronounced macrocytosis and a moderate anemia and achlorhydria is significant, and it is agreed with other investigators that swine may be a better animal in which to produce experimentally a pernicious anemia-like syndrome under controlled conditions.

Summary and conclusions

1. Hematological observations have been made on two swine for approximately seventeen months after exposure to atom bomb ionizing radiation at Bikini.

2. Macrocytic anemia and achlorhydria were found in both animals and a hyperplastic, "megaloblastic-like" bone marrow in one.

3. The experimental background of these swine with special reference to nutrition, radiation, age, and disease has been considered. However, it was not possible to evaluate conclusively the relative role that these conditions played in the production of the anemia and associated bone marrow findings.

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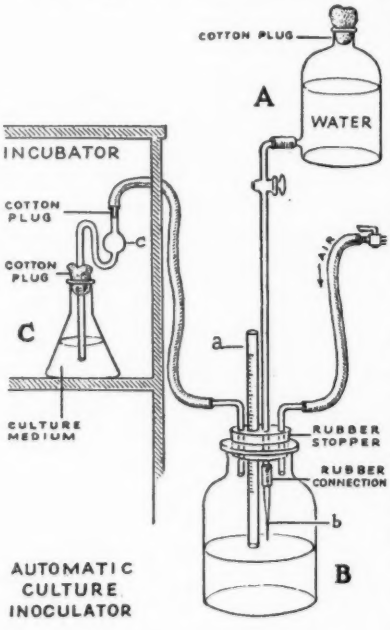
A SIMPLE APPARATUS FOR THE AUTOMATIC INOCULATION OF CULTURE MEDIA†

ROBERT A. GOLDWASSER*

In connection with certain investigations being carried out in this laboratory, it was found necessary to work with six- to twelve-hour cultures of bacteria in fluid culture media. The apparatus described below was devised to obviate the need of either coming to the laboratory late in the evening to inoculate cultures for the following day's work or of wasting time waiting for cultures to grow out after inoculation in the morning.

Since the author was unaware of any description in the literature of a simple apparatus for automatically inoculating a culture at any predetermined time and since it was felt that there exists a need for such an apparatus, it was decided to present the one devised in this laboratory.



Description of the apparatus

As shown in the diagram below, the apparatus consists of the following: A 3 to 4 liter bottle (A) with a sidearm which is connected by means of a glass tube to a second bottle (B). Bottle A rests on any convenient bench or stand, and bottle B rests on the floor. The tube connecting A and B terminates in a piece of glass tubing drawn out to a fairly fine tip (b). The opening in tip (b) is fine enough to deliver a slow, steady stream of water at a rate which can easily be determined (1.5 ml. per minute is used in this labora-

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tory). A tube (a), which is open at either end, is inserted through the rubber stopper in bottle B. The tube (a) is calibrated according to the volume of B, so that its lower end will just make contact with the surface of corresponding amounts of water in B. Two more tubes, one serving as an inlet and the other as an exit of air, are passed through the rubber stopper of B. The inlet tube is connected to any convenient source of positive air pressure. To the exit tube is connected rubber tubing which passes through any convenient opening in the incubator. This tube is further connected to a piece of glass tubing (c) with a double U bend which may have a bulb blown in its upper end. Two ml. pipettes (the ends of which had been chipped and which therefore were discarded) were found to be quite satisfactory for this purpose. Tube (c) is passed through a cotton plug into flask C, which contains the culture medium. (For flask C, an ordinary test tube may be substituted if smaller amounts of culture are desired.)

Operation of the apparatus

Tube (c), which has previously been sterilized, is inserted with aseptic precautions through the cotton plug of the flask containing sterile medium. The fluid inoculum is transferred to tube (c) with the aid of a capillary pipette. Care must be taken that the level of the inoculum does not rise above the level of the upper U bend, since if it does it will siphon over into the medium. The cotton plug in C is replaced as a precaution against contamination of the inoculum, and the rubber tube entering the incubator is connected to (c). Bottle A is then filled with water, preferably filtered or distilled since particles in the water would clog tip (b). A simple calculation is then performed to determine the amount of water which will have flowed from bottle A to bottle B at the time at which inoculation is desired to take place. For example, if tip (b) delivers 1.5 ml. per minute and inoculation is desired ten hours after the apparatus is set into operation, 900 ml. of water will have drained by that time into bottle B. Tube (a) is then adjusted so that its bottom opening will just contact the surface of the water in B after the desired volume of water has entered. The stopcock allowing water to flow from A to B is then opened, and the air pressure is turned on. (Only a very slight pressure is necessary.) The air will escape through (a) until the water level in B rises enough to contact the lower opening of (a), when the air will be forced to escape through tube (c), forcing the inoculum into flask C. If aeration of the contents of C is desired, (c) may be adjusted to project below the surface of the medium; otherwise it may be held, by the cotton plug in C, above the surface.

After tip (b) has been calibrated to deliver a known amount of water per minute, only one adjustment need be made thereafter in the apparatus, namely the length of tube (a), which projects into bottle B. Since (a) has been calibrated to contact known levels of water in B, this adjustment is simply performed.

In order to re-use the apparatus, the rubber stopper in B with its attached tubes, together with unit A, can be lifted out and moved aside while bottle B is emptied. The whole unit can thus be disassembled in a single operation and likewise reassembled.

In this laboratory it has been found that reasonably accurately timed inoculation can be performed with the above apparatus even when it is put in operation at Saturday noon and set to inoculate as late as Monday morning.

CATHERINE STRUNK AMATRUDA

The sudden death of Catherine S. Amatruda, M.D., Assistant Professor of Pediatrics at the Yale University School of Medicine, on September 1, 1949, came as a profound shock to her circle of family, friends, and associates. Dr. Amatruda, born in 1903, received her collegiate training at Cornell University, where her father was a professor, and her medical training at Cornell Medical College. For eighteen years she was a staff member of the Clinic of Child Development at Yale University, in 1948 receiving her present appointment in the Child Study Center at Yale University. She was an associate pediatrician of the Grace-New Haven Community Hospital, a specialist certified by the American Board of Pediatrics, a fellow of the American Academy of Pediatrics, and a member of a small and distinguished group — the American Cerebral Palsy Association. Dr. Amatruda was married and had a son and a daughter. Her death followed the spontaneous rupture of a cerebral basilar aneurysm.

Dr. Amatruda's special interests included cerebral injury and handicapped children—the cerebral palsied, the deaf, the blind, the mentally defective. She was interested in the general improvement of adoption proceedings and played an active part in the enactment of improved adoption laws in the state. She is the co-author of the book, *Developmental Diagnosis*, and collaborator in other volumes dealing with infant and child development.

As a person, Dr. Amatruda had charm and equanimity; she respected the dignity of the individual. As a physician, she was unselfish and devoted; as a medical scientist, she showed outstanding and exceptional abilities. As a teacher, she preferred small groups and here her ability and brilliance were unsurpassed. Those who were fortunate to have Dr. Amatruda as their associate, knew her also as a completely sincere and trustworthy friend of unequalled merit.

JOSEPH V. BALDWIN

ADAPTATION TO BRIEF STRESS*

The Blood Level of Leucocytes and Adrenal Function in Epilepsy, Electrically Induced Convulsions, and after Injection of Epinephrine. A Review and Interpretation.

STANLEY T. MICHAEL

The time-honored, useful, and frequently applied laboratory test of clinical medicine—the white blood cell count—is acquiring new meaning through recent discoveries in the basic sciences of medicine. The observation by Dougherty and White,[16,17,19] and by Reinhardt, Aron, and Li,[58] of the dissolution of lymphocytes by the action of adrenocortical hormone provokes reinterpretation of a number of physiological and pathological phenomena which have long been known to be accompanied by changes in the level of circulating leucocytes. The following discussion will be focused on an interpretation of the leucocytic changes which occur during brief stress.

The adrenal cortex and its secretory activity are important factors in the adaptation of the total organism to stress situations.[63] A great variety of stress situations such as exposure to cold, exhaustive physical effort, burning, scalding, injuries, hemorrhage, or infections induce activity of the adrenal cortex, with consequent metabolic changes which have been organized by Selye[66] into a concept of the "adaptation syndrome." The variety of stress agents which provoke function of the adrenal cortex prompted Long[49] to search for a common factor involved in all stress situations. He predicted this factor to be secretion of epinephrine and was able to support his concept experimentally by producing the adreno-cortical changes of stress by injection of appropriate doses of epinephrine. The physiological changes of adaptation induced by stress or the injection of epinephrine do not occur in hypophysectomized rats. Epinephrine, therefore, does not directly affect the adrenal cortex. Long postulates that the adreno-cortical mechanisms of adaptation are initiated by secretion of epinephrine, which in turn induces pituitary secretion of adreno-cortico-trophic hormone with resulting secretion of adreno-cortical hormones and consequent adaptation phenomena.

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The stress situations which are associated with adreno-cortical function tend to be of prolonged, chronic quality. In contrast to the extended, enduring stress situations, an acute, intense, violent type of stress condition, accompanied by outpouring of epinephrine or epinephrine-like substances from the medulla of the adrenal gland has been established by the older observations of Cannon[10] and his school. Evidently both types of hormones of the adrenal gland have essential functions in stress situations.

An attempt will be made herewith to gather observations from the extensive literature dealing with changes in blood levels of leucocytes during stress situations to determine whether the blood leucocyte changes have meaningful relation to the known functions of the adrenal cortex. The study will be restricted primarily to stresses of short duration, and to the study of leucocyte levels after injection of epinephrine. From the standpoint of method, the phenomena following injection of epinephrine are a controlled experiment and will be therefore discussed first as a quasi prototype. The leucocyte changes following electrically induced convulsions, epileptic convulsions, intense brief exercise, and emotional stress will be then compared with the model pattern of epinephrine.

Epinephrine and adreno-cortical hormones

As mentioned in the introduction, Long presented evidence that epinephrine can duplicate the effects of stress situations on the adrenal cortex and produce internal secretion of cortical hormones. Cortical hormones, in turn, cause dissolution of lymphocytes in mice[16] and men.[26, 42] On the basis of these observations alone, one would be led to expect a decrease in circulating lymphocytes following injection of epinephrine. In actuality, injection of epinephrine produces an immediate, absolute, and relative increase in circulating lymphocytes. The priority of this observation is attributed to Harvey,[38] and the literature is replete with protocols which confirm this observation. Since there is usually also an increase in the absolute number of circulating neutrophils, the net result is an increase in the total leucocyte count. The maximum increase in cells occurs usually between 15 and 45 minutes following injection of epinephrine.

The protocols of the few observers who extended their epinephrine studies beyond the arbitrary one-hour limit, which seemed to appeal to the great majority, show a subsequent fall in lymphocyte count with a relative and absolute neutrophilia. The occurrence of two phases of the lymphocytic response to epinephrine injection was first described in rabbits and guinea pigs by Frey[27] and subsequently found also in human subjects.[28, 32] While

the older observers were unable to explain the second phase of lymphopenia, it seems now possible to attribute this phase to the action of adreno-cortical hormone,[16, 17, 19, 58, 59] whose secretion was initiated by epinephrine, through stimulation of the pituitary corticotrophic hormones as postulated in the scheme of Long.[49] Injection of adreno-cortico-trophic and adreno-cortical hormones causes not only lymphopenia, but also a marked increase, relative and absolute, of polymorphonuclear cells.[16, 26]

It is interesting to note that the investigators of our present era had their predecessors. Bertelli, Falta, and Schweeger[5] injected dogs with anterior pituitary hormone. Their protocols demonstrate, both through a decrease in lymphocytes and an increase in neutrophils, that the injected material contained potent adreno-cortico-trophic hormone. The observation of Bertelli and co-workers is all the more significant in its support of adreno-cortical leucocytic relationships since it was made unintentionally, and the results admittedly did not fit into the scheme of interpretation of leucocytic mechanisms forwarded by the authors.

The opposite of the injection experiment—the extirpation experiment—is also available to support the concept of the influence of the adrenal gland on circulating neutrophils. Borchardt[7] demonstrated that it is impossible to induce a neutrophilic leucocytosis in adrenalectomized cats by the usual methods, such as injection of epinephrine, pilocarpine, choline, by infection, or by suffocation.

The second phase of the epinephrine reaction—the phase of lymphopenia —obligingly supports the theoretical assertions as to its causation, also through a characteristic and fairly consistent increase in circulating neutrophils. The sequence of events which results from injection of epinephrine may then be considered in two phases: Phase I, characterized by lymphocytosis, and Phase II, characterized by lymphopenia and an increase in circulating neutrophil cells. Phase I is probably a result of direct action of epinephrine—the epinephrine phase proper; Phase II, an expression of the adreno-cortical phase of the epinephrine reaction.

Most of the literature which deals with Phase I—the "adrenalin lymphocytosis"—is preoccupied with the source of the lymphocytes which appear in the blood stream to swell the total number of leucocytes. A substantial portion of the research in connection with the epinephrine lymphocytosis was stimulated by W. Frey's consistently reiterated assertion that the spleen was the most important source of the adrenalin lymphocytosis, and his promotion of the phenomenon as a diagnostic test for illnesses of

the spleen. The publications on the source of the "adrenalin lymphocytosis" were reviewed by several authors.[3, 20, 39, 43, 45, 54]

Most investigators conclude that the epinephrine lymphocytosis is a result of composite action of several factors which may be generally divided into a group of mechanical, physical factors, and a group of chemical factors. Although a number of aspects is involved, the mechanical factor of redistribution of lymphocytes from locations of immobilization and storage into the blood circulation, and secondly, a factor of adreno-cortical function probably account for the major proportion of the lymphocytosis.

As mentioned above, contraction of the spleen contributes an important portion of the "adrenalin lymphocytosis." Lymphocytes are also added to the blood stream from lymph nodes, as may be observed from a markedly increased flow of lymph and lymphocytes through the thoracic duct.[2, 56, 61, 72] Small blood vessels contain lymphocytes and other leucocytes, which in the resting state attach themselves to the walls of the dilated blood vessels under conditions of stagnation of the blood stream. Constriction of the blood vessels and increased rapidity of circulation due to action of injected epinephrine sweep the attached cells into the blood stream.[9, 21, 45]

There have been no direct studies to prove the involvement of adreno-cortical factors in the epinephrine lymphocytosis; however, the related evidence more than implies the significance of an adreno-cortical agent. It is known that conditions in which the production of adreno-cortical hormones is increased, such as Cushing's syndrome, are characterized by relative lymphopenia.[14] In contrast, Addison's disease, a condition of decreased adreno-cortical function, is characterized by lymphocytosis.[14, 48] These clinical observations are supported by animal experiments. Adrenalectomy in cats,[74] rats, and dogs[13] also leads to marked lymphocytosis, thus giving confirmation to the observation on patients with Addison's disease. Corey and Britton[13] were able to restore the pre-operative blood picture in adrenalectomized animals by administration of adreno-cortical hormones. Increased levels of corticosterone thus lead to greater dissolution of lymphocytes and lymphopenia, while the converse is true of decreased levels of corticosterone which lead to lymphocytosis. Sayers and Sayers[62] were able to show that increasing the blood level of cortical hormone by pretreatment of rats with corticosterone prevents the usual metabolic manifestations of adreno-cortical function. It is possible to surmise that injection of epinephrine, or "participation in a stress situation might cause in the tissues utilization of cortical hormone, thus lowering the level in the blood,"[49] which in turn would lead to lymphocytosis. The adrenalin lymphocytosis of

Phase I may thus be in part due to decreased corticosterone function, as well as to the mechanical factors enumerated above. An observation of Behr[3] lends support to this contention. Behr observed the usual lymphocytosis after contraction of the spleen following injection of epinephrine; however, when he stimulated the exteriorized spleen to contraction by a faradic current, no lymphocytosis followed the contraction of the spleen.

Clinical application of the epinephrine lymphocytosis reaction as a critical diagnostic test for diseases of the spleen, especially Banti's disease, was pioneered and staunchly advocated by W. Frey and his co-workers. He reported an average increase of 3,300 lymphocytes to be normal. An increase above 2,500 lymphocytes per cmm. is to be considered normal. An increase of less than 1,500 was considered pathognomonic of disease of the spleen.[29, 30] According to Schenk,[65] epinephrine causes an increase of the lymphocyte count comprising 14-24% of the total leucocyte count. Friedberg[32] found an average increase of approximately 4,000 lymphocytes one-half hour after subcutaneous injection of epinephrine in a group of 51 children.

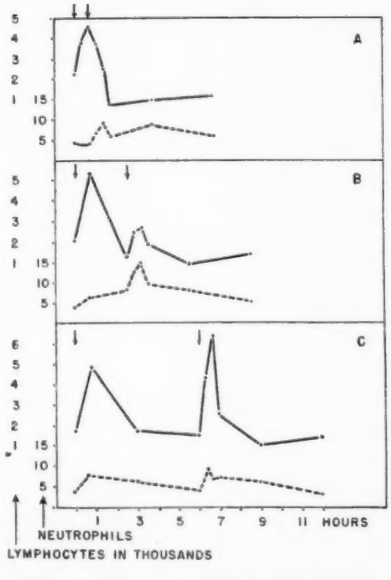

FIG. 1. Responses of blood lymphocytes after repeated subcutaneous injections of epinephrine. Injection of epinephrine indicated by arrow. Reconstructed from data by Hatiegan.[30]

It soon became obvious that the epinephrine lymphocytosis failed to occur in disease conditions other than Banti's disease and splenic fibrosis. Thus Frey and Lury[30] observed failure of the epinephrine lymphocytosis in a case of scleroderma. It is also absent in typhoid fever,[40] influenza, sepsis due to staphylococci, anaphylaxis, one day or 16 days after injection of horse serum,[31] acute encephalitis, and irradiation by roentgen rays.[4] Walterhofer[69] observed failure of the epinephrine lymphocytosis also in normal subjects. In a group of 68 subjects, Lucia *et al.*[50] observed a leucocytosis in 64 cases, while an early relative lymphocytosis occurred in only 56 subjects tested.

By ingenious experiments Hatie-

gan[39] demonstrated that injection of epinephrine itself may prevent a subsequent epinephrine lymphocytosis over a period of 6 hours. If epinephrine is injected 40 minutes following a previous injection of epinephrine (see Fig. 1) during the height of lymphocytosis produced by the first injection, it causes no further increase in lymphocytosis and the lymphocytes continue in their pattern of decrease as if the second injection were not given. If the second injection of epinephrine is given three hours after the first one, there is only a slight lymphocytic reaction, and instead there is apt to be a more significant increase in neutrophils. If the second injection of epinephrine is given after six hours, the usual epinephrine lymphocytosis recurs. In the light of our present knowledge it may be surmised that the failure of the typical epinephrine lymphocytosis after a previous injection of epinephrine is due to action of adreno-cortical hormone which had caused dissolution of lymphocytes and thus depleted the available reserve of lymphocytes which would otherwise be mechanically swept into the blood stream.

A similar interpretation applies to other conditions in which epinephrine lymphocytosis fails to occur. Thus acute infections are a chronic stress situation accompanied by adreno-cortical activity.[66] Similarly, irradiation by roentgen rays induces function of the adrenal cortex.[18] The failure of epinephrine lymphocytosis under the conditions quoted above may be interpreted as an indication of increased adreno-cortical function on the same basis as the failure of epinephrine lymphocytosis 3 hours following a previous injection of epinephrine. Conversely, a marked lymphocytosis following injection of epinephrine indicates by inference a low level of corticosterone function and a consequent large number of readily available lymphocytes in storage reservoirs. A further probable prerequisite for a sizeable epinephrine lymphocytosis is relaxation of blood vessels and storage spaces, and stagnation of lymphocytes.

Since both circulating neutrophils and lymphocytes are influenced by adreno-cortical hormone, inclusion of both variables in a single value, as is the case in the differential blood count, makes the value a more specific indicator of adreno-cortical function. A still more specific measure of adreno-cortical function, a ratio involving only lymphocytes and neutrophils, L/N, will be discussed under the title, "Comments."

The duration of the change in the blood leucocyte picture is probably not limited to the two or three hours suggested by the majority of investigators, and not even to the six hours claimed by Hatiegan. Friedberg[32] states that at least twice twenty-four hours should elapse between experiments, as he

had noticed a polymorphonuclear leucocytosis extending over a protracted period of time following an injection of epinephrine. Loeper and Crouzon[48] presented graphs as evidence that the injection of one mg. of epinephrine in the human subject causes distortion of the blood leucocyte picture over a period of four days. The mechanism of this type of extended reaction and its physiological significance is not clear. The authors fail to give experimental conditions of their observations, which also beclouds the issue. Observations of prolonged action of epinephrine must also take into account the possibility of a production of leucocytosis on the basis of a conditioned reflex mechanism in relation to the sampling procedure.[64] Also emotional attitudes to the sampling procedure must be considered, as it has been shown that both a conditioned reflex mechanism and disturbed emotions may cause a leucocytosis.[70, 74] These conditions apply to human beings as well as to animals.

The duration of the change in the blood leucocyte picture induced by the stimulus of a single injection of epinephrine probably has some relation to the dose of epinephrine and also to the circumstances of injection and sampling. While emotional factors may play a role with small doses of epinephrine, it is probable that the large doses ranging from 3 to 9 mg. injected into dogs by Bertelli, Falta, and Schweeger,[5] were responsible on their own bio-chemical merit for the production of responses extended over a period of three or four days.

When a given dose of epinephrine is repeated at daily intervals, both rabbits and human subjects respond at first by a leucocytosis. However, subsequently the organism adapts to the dose of epinephrine and no leucocytosis occurs. A steadily increasing shift in the blood picture toward a relative lymphocytosis may be observed.[48]

Walterhofer[69] and later Behr[3] injected animals with repeated doses of epinephrine over a protracted time and then sacrificed them to study their bone marrow. Both authors reported alteration in the structure of the bone marrow indicative of increased hematopoietic activity. The mode of stimulation of the hematopoietic organs is not at all clear and has been attributed for instance to anoxemia of the bone marrow due to contraction of blood vessels caused by the action of epinephrine.[69] Since it is now known that injection of epinephrine induces adreno-cortical function, it would seem reasonable to ascribe the alleged hypertrophy of bone marrow after repeated injections of epinephrine, and the neutrophilia observed after a single injection of epinephrine, to a stimulative action of adreno-cortical substances on

myelogenous structures. That a hyperplasia of myeloid elements does occur after adreno-cortical secretion has been observed by Dougherty and White.[17]

Electrically induced convulsions

Convulsions artificially induced for the purpose of treatment of patients with mental illness are representative examples of stress of short duration in human subjects. These convulsions are accompanied by severe muscular action, apnea, and frequently cyanosis. The average duration of 326 electrically induced convulsions was found to be 41.5 seconds.[25] The blood leucocyte aspects of electric convulsions are characterized by an initial lymphocytosis[12, 24, 52] which subsequently recedes.[24, 52] A similar pattern occurs in convulsions induced by metrazol.[6, 23] The similarity in the same individual of the epinephrine lymphocytosis and the lymphocytosis of an electric convulsion is evident from three representative examples rendered in Fig. 2.

The close similarity between the epinephrine response and the response after an electric convulsion applies only to the first electric convulsive treatment. With subsequent convulsions the immediate increase in lymphocytes becomes less pronounced and may be altogether absent 20 minutes after a convulsion. As the number of convulsions increases, there is a definite shift of the pre-treatment leucocyte levels toward relative lymphocytosis in some patients. The relative lymphocytosis is usual-

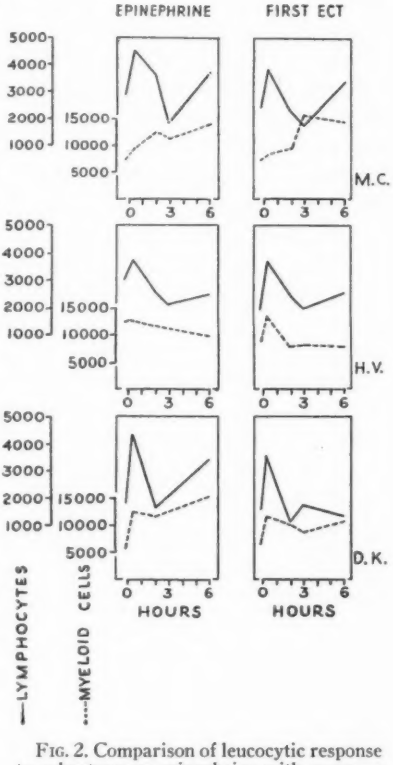

FIG. 2. Comparison of leucocytic response to subcutaneous epinephrine with response to a single electric convulsive treatment (E.C.T.). Epinephrine 1 mg. per 70 kgm. body weight, preceded E.C.T. by 72 hours in subject M.C., 48 hours in subject H.V., and 24 hours in subject D.K. Myeloid cells include neutrophils, eosinophils, and basophils.

ly well pronounced after ten or more convulsions. With increasing numbers of convulsion, the second, the adreno-cortical phase of the leucocytic response may become more pronounced in individual subjects. The increased intensity of the adreno-cortical phase is not always evident from a decrease in the lymphocyte level, which, in some instances, may show little variation after an electric convulsion, but rather, from combined observations of both the lymphocyte and neutrophil levels, as for instance, in the relative lymphocyte counts. It is at times possible to observe at the two-hour interval in successive convulsions, an irregular alteration of a pattern of lymphopenia accompanied by little change in the neutrophil count with a pattern of marked neutrophilia and little change in the lymphocyte count.

The observations dealing with the leucocyte changes following electrically induced convulsions are presented in greater detail elsewhere.[52] Suffice it to emphasize here that the initial stress pattern is similar to the pattern following epinephrine injection, and that progressive repeated convulsions lead to a progressive cumulative change in the blood leucocyte formula indicating probably a progressive change in adrenal function.

Epilepsy

The stimulus which leads to the changes in the level of circulating lymphocytes discussed in the previous sections on epinephrine and electric shock therapy was applied from the environment. The stimulus was controllable not only as to the magnitude of dose but also in respect to time. The timing of the stimulus is especially important in studies of brief stress, as it makes it possible to relate the various observed phenomena to each other and to a fixed, original causative agent. In contrast, in epilepsy, the stimulation of the central nervous system which leads to a convulsive seizure is endogenous, originating from within the disturbed metabolism of the subject.

The endogenous nature of the stimulus in epilepsy is a source of difficulty in the study of reactions to the convulsions. The most distressing factor is probably the unpredictability of the oncoming convulsion. In sporadically occurring convulsions it is a fortunate accident when a control sample is obtained shortly before the onset of the epileptic convulsion. The problem of predictability of the onset of the epileptic convulsion has probably resulted in weighting of the literature with protocols of cases which have a protracted aura. It is probable that the selection of cases was influenced by this factor which affords the investigator sufficient time to collect control samples before the onset of the convulsion. Since states of excitation of the

central nervous system, especially states of emotional anxiety, influence the blood leucocyte level in the same manner as does the convulsion proper, the results obtained from the study of epileptic convulsions with an aura are not necessarily representative of all epileptic convulsions.

To cope with the unpredictability factor, most investigators have approached the problem by taking daily or periodic blood counts and thus establishing a base line as a control sample.[15, 36, 37, 71] Convulsion samples were then collected whenever the convulsion occurred. By chance, an occasional routine sample was collected shortly before a spontaneous convulsion. A large proportion of protocols presented in the literature are derived from patients with frequent convulsions, although the excellent work of Krumbmiller[46] includes protocols of patients with intervals between convulsions ranging over several months.

Despite the obvious difficulties of methodological approach, the variability of the studied cases, and the individual discrepancies in interpretation of results by various authors,[35, 47] the recorded data are fairly uniform in their amenability to interpretation along the herein developed concepts of adrenal mechanisms of leucocytic responses. Most observers who studied the leucocytic responses to epileptic convulsions are in agreement on the occurrence of leucocytosis following convulsions.[8, 11, 15, 36, 37, 43, 44, 46, 57, 60] Samples collected immediately following the convulsion usually show lymphocytosis. Samples collected after intervals longer than thirty minutes are usually characterized by relative lymphopenia and neutrophilia. The typical pattern of leucocytosis also occurs after convulsions resulting from known etiological factors, such as dementia paralytica or other organic brain disease.[8, 11]

A topic of controversy revolves about the question as to what happens preceding an epileptic attack. Itten[44] and Zimmerman[73] report an increase in the total leucocyte count with a relative lymphocytosis which begins one to two days preceding an attack. Itten indicates that following an attack the leucocytosis recedes to the lower value of the attack-free interval only if no other attacks follow within 48 hours. However, if the leucocyte count continues high after an epileptic attack, another attack occurs within 24 to 36 hours. Di Gaspero[15] observed a leucopenia with lymphocytosis several hours preceding an attack and ventured to discuss predictability of an attack on the basis of the leucopenia. Di Gaspero stresses that the decrease in leucocyte count is due principally to a decrease in neutrophils. He also claims that there is a tendency to relative lymphocytosis in the interparoxysmal period. Krumbmiller,[46] by repeated observations on one case with regular daily attacks, documents a normal blood picture up to six

minutes preceding an attack. The protocols presented by the various authors show that their conclusions were derived from individual cases rather than from large numbers of observations—a natural consequence of the technical difficulties mentioned above.

Our own studies with electrically induced convulsions[52] seem to indicate that all three variables of lymphocytosis, lymphopenia, or no change in distribution of leucocytes may occur. The comparison is not strictly parallel as the endogenous excitant in epilepsy which leads up to a final peak of excitation and a seizure is probably exerting an influence on the blood counts for an indefinite interval preceding the seizure. In contrast, in electrically induced convulsions, any influence of a seizure on the blood count must be attributed to a preceding seizure. The prodromal anticipation of an epileptic, especially in the presence of an aura, and the emotional anticipation of the patient who is about to receive an electric convulsive treatment are possibly comparable factors in both conditions.

In 13 patients who received a total of 113 electric convulsive treatments and therefore had 100 multiple convulsions, the relative lymphocyte count was above the first control levels in 57 instances preceding the multiple treatments and lower in 38 instances, while on 5 occasions the relative lymphocyte count was approximately the same as preceding the first treatment. However, the increase in lymphocytes was equal to or exceeded 10% of the total neutrophil count in 35 instances, while the decrease equaled or exceeded 10% of the total neutrophil count in only 9 instances. An increase or decrease in lymphocytes of 10% of the neutrophil count or 0.1 L/N represents 500 lymphocytes if the neutrophil count is 5,000. As evident from these values there is a greater tendency toward lymphocytosis than toward lymphopenia in serial convulsive seizures.

These proportions in a tendency toward lymphocytosis or lymphopenia are also borne out by the statistical studies of Spangler.[67] This author reported lymphocyte counts above normal range in 51%, below normal range in 10% of his 100 epileptic cases. Fifty-two per cent of the 182 cases of Patterson and Weingrow[55] showed a leucocytosis—predominantly lymphocytic—while 20.3% showed a leucopenia. Zimmerman[73] considered normal the average leucocyte count of his 10 epileptic subjects during an attack-free period, although a relative lymphocytosis was observed in three subjects.

The predisposition to have a relative lymphopenia or leucopenia as contrasted to the predisposition to have a relative lymphocytosis during the emotional tension preceding an electric convulsive seizure may be an

expression of the constitutional structure of the personality of the patients. Since a detailed report will be rendered elsewhere,[52] it will be only mentioned here that multiple electric convulsions of those individuals who are active or excited are preceded by increasing lymphopenia as their condition improves. In contrast, depressed, underactive patients are apt to show an increasing lymphocytosis concomitant with improvement in their depressed condition.

The frequency, severity, and character of the convulsions are an important factor in determining the type of reaction of the blood leucocytes. Thus it is obvious from the protocols of several authors, [37,43] but clearly demonstrated in the excellent graphs of Wuth[71] that a series of multiple convulsions occurring within a period of a few days leads to a marked leucocytosis with relative lymphopenia which gradually adjusts to normal values over a period of three or four days following the last convulsion. In contrast, in the case presented in Chart 8 of Wuth,[71] one convulsion daily over a period exceeding two weeks led to a persistent relative lymphocytosis in a manner similar to that observed in some cases of electric convulsive therapy[52] and also after multiple injections of epinephrine.[48]

An interval of three or four days before or after a seizure is generally accepted as necessary for indication that a patient's leucocyte count is free from the influence of a seizure.[55] This observation is of interest in connection with the empirically derived spacing of convulsions induced electrically in mental patients for therapeutic purposes as the majority of therapists produce therapeutic convulsions at two- to three-day intervals.

In summary, the observations pertaining to changes in leucocyte counts during epilepsy relate to two types of phenomena: (i) The brief stress situation of the convulsion and the resulting conditions which appear within a few hours following the brief stress situation; (ii) the overall leucocytic changes related to the epileptic condition and extending over periods of days or possibly over the whole duration of the epileptic condition.

The majority of the well-documented observations relating to the pattern of blood leucocyte change immediately following an epileptic seizure are in accord with the observations pertaining to electrically induced convulsions and confirm the general pattern occurring after an injection of epinephrine. The epileptic convulsion is, in general, followed by leucocytosis which is at first lymphocytic and in its second phase neutrophilic. This pattern is not readily evident in convulsions which occur at short intervals measured in terms of minutes and hours. Even in this respect of a less pronounced leucocytic response there is similarity between multiple spontaneous con-

vulsions and multiple induced convulsions. The failure of lymphocytosis after multiple, closely spaced injections of epinephrine is thus also matched by the failure of lymphocytic responses following multiple convulsive phenomena.

The leucocytosis in epilepsy which has attracted the attention of observers during the past fifty years may be classified in generalization as:

1. Interparoxysmal lymphocytosis occurring in occasional cases.
2. Prodromal lymphocytosis preceding attacks by 24 to 48 hours in some patients.
3. Convulsion lymphocytosis occurring within a few minutes after an epileptic attack in most patients.
4. Post-convulsion leucocytosis, usually neutrophilic which persists for several days following a convulsion.

Exercise

The review of the literature on leucocytosis in exercise presented by Garrey and Bryan[33] provides sufficient information to enable one to draw the conclusion that the pattern of leucocytosis following exercise does not differ essentially from the pattern of leucocytosis occurring after a convulsion or the injection of epinephrine. Short bouts of severe exercise cause an immediate lymphocytic leucocytosis which soon gives way to a polymorphonuclear leucocytosis. Leucocyte counts after a marathon run which lasts between two and a half and three hours show a marked increase in polymorphonuclear cells which in one study formed between 84.4% to 91.4% of the total leucocyte count. It was observed that the neutrophilic leucocytosis "did not subside to the normal count after 24 hours."

Several authors have also noticed that in trained subjects there is only little distortion of the leucocytic formula following exercise. Evidently the leucocytic response to exercise may be absent in a manner similar to the absence of the leucocytic response observed after repeated doses of epinephrine or after repeated convulsions occurring at short intervals.

Emotional leucocytosis

A number of investigators have provided evidence of an association between conditions of psychic excitation and the blood leucocyte count. Pertinent references were gathered by several reviewers.[33, 43, 53] In the discussion of brief stress situations the emotional leucocytosis again poses a problem similar to that of epilepsy in the diffuse and inaccurate timing of the stimulus. Probably the most suitable observations in this respect are those of Wittkower,[70] Bath,[1] and Menkin.[51]

Wittkower[70] suggested states of joy, sadness, anxiety, rage, or jealousy under hypnosis and observed increases of 2,000 to 5,000 in the leucocyte count and a "mild lymphocytosis." Bath,[1] in her studies of leucocytosis following a meal, noticed that a prompt leucocytosis occurred even if the meal were merely placed in front of the subject without subsequent ingestion. This type of experiment presents some of the finer aspects of emotional leucocytosis, as the leucocytosis might also be interpreted as developing on the basis of a conditioned reflex.[64] A state of emotional "mononucleosis" was observed in cats by Menkin.[51] A consequent decrease of the mononuclear cells below the initial level occurred 40 minutes after the emotional stimulus.

States of prolonged excitation, especially those which accompany mental illness, are accompanied by a protracted leucocytosis. The type of leucocytosis is a matter of contradiction in the various reports in the literature. Hoff[43] in his review states that a neutrophilic leucocytosis prevails during the state of excitation. During remission from excitation a lymphocytic leucocytosis succeeds the neutrophilic leucocytosis in the same manner as following infectious illnesses. Tinel[68] also observed a neutrophilic leucocytosis in states of manic excitement or anxious agitation. In contrast, Itten[44] reports and documents by graphs that a lymphocytic leucocytosis prevails during the morbid stage of schizophrenia. If the schizophrenic improves, an accompanying abatement of the lymphocytic leucocytosis ensues. Farris[22] observed a protracted lymphocytosis during emotional tension and anxiety preceding final examinations in medical students. The lymphocytosis was also sustained over the two-day period of examinations. The emotional lymphocytosis receded two to five hours after an examination following which the subjects were reassured of passing. Milhorat[53] and co-workers, also Hill,[41] found no change in the differential blood count in states of chronic excitation and anxiety.

Comments

Survey of the literature dealing with blood leucocyte levels and their variation points to the conclusion that brief states of excitation of the human and higher vertebrate organisms lead to typical changes in concentrations of circulating leucocytes. Function of the intact vertebrate organism can not be conceived as devoid of simultaneous involvement of the nervous system; nevertheless a typical response in the leucocyte picture occurs regardless whether emphasis of the excitation is placed on the muscular system, the digestive system, or on the function of the psyche. The same

pattern of leucocyte change occurs after muscular exercise, a convulsion, administration of food or drugs, or after psychic stimulation. Under specified conditions—the most important condition being absence of a stress situation preceding the observed excitation—the leucocyte change consists of a lymphocytic leucocytosis immediately following the excitation. After approximately one hour the lymphocytosis gives way to a reverse situation —a lymphopenia with a neutrophilic leucocytosis. Extirpation, injection, or substitution experiments point to the adrenal gland and its function as the agent responsible for the typical leucocytic response. The master pattern for all conditions which invoke the leucocytic response is the pattern which follows injection of epinephrine.

De la Balze *et al.*[14] compared the relative lymphocytosis of Addison's disease with the lymphopenia observed in Cushing's syndrome. While the absolute counts of lymphocytes per cu.mm. overlapped to a slight degree in the two disease conditions, the relative counts of lymphocytes per 100 cells showed no overlap whatsoever. De la Balze and co-workers concluded that the differential blood count was a reliable indicator for differentiation of Addison's disease from Cushing's syndrome.

Closer analysis of the data presented by de la Balze and co-workers reveals that the greater resolving power of the relative blood count is due to mathematically relating the lymphocyte count to the neutrophil count, which in Cushing's syndrome was on the average twice as high as in Addison's disease. The mathematical relation of the percentage of lymphocytes to that of the neutrophils is based on limitation and relation of both variables to 100 cells counted—as expressed in the formula:

$$L + N + X = 100$$

where L equals lymphocytes per cent, N equals neutrophils per cent, and X equals the sum of the remaining white cells per cent. In this equation, any increase in N results in a depression of L and X, due to the fixed ratio to 100 cells. Thus, for instance, using values from protocols of de la Balze and associates, a count of 2,068 lymphocytes per cubic millimeter in a patient with Cushing's syndrome and a neutrophil count of 16,544 resulted in a relative lymphocyte count of 11%.

In order to use the leucocyte count as a numerical expression of adrenocortical function, two conditions should be fulfilled. It is first desirable to eliminate non-specific factors.[34] While this is not wholly achievable, it is possible to eliminate the non-specific factor of X in the equation $L + N + X = 100$, mentioned above, by the non-inclusion of the value of X in the desired formula. With the exception of eosinophil cells, which

decrease in number with an increase of adreno-cortical function,[26] the value of X in the above equation is not related to adreno-cortical function.

Secondly, since the value of L and N is influenced in opposite directions by adreno-cortical function, it is desirable to place them in a reciprocal, mathematical relationship. A formula which satisfies both conditions is a simple ratio of the lymphocytic count to the neutrophil count—L/N—which thus makes the lymphocyte count an indirect function of the neutrophil count. Since the total lymphocyte count per cu.mm., and the total neutrophil count per cu.mm. are derived in both instances by multiplication of the total leucocyte count by the percentage of the individual types of cells,

$$\frac{L\% \times \text{total leucocyte count per cu.mm.}}{N\% \times \text{total leucocyte count per cu.mm.}}$$

it is possible to derive the ratio L/N from the differential count alone, as the total leucocyte count is eliminated by cancellation.

The advantage of the ratio L/N as a numerical expression of adreno-cortical function may be gleaned from the following table in which the previously mentioned values of de la Balze *et al.* derived from patients with Cushing's syndrome and Addison's disease are compared.

	Lymphocytes per cu.mm.	*Neutrophils per cu.mm.*	*Lymphocytes Per cent*	*L/N*
Addison's disease	2,016	5,380	24	.37
Cushing's syndrome	2,068	16,544	11	.13

Obviously, changes in the level of circulating leucocytes are not the only physiologic manifestation which follows a brief stress situation. Evidence from a wealth of publications points to the involvement of many biochemical constituents in the metabolic commotion which follows brief stress.[60, 63, 66] The pattern of the metabolic changes and the time intervals, which are similar to those of the leucocyte changes, direct attention to the adrenal gland as the governing force of many aspects of the dynamic sequence of adaptation to the stress situation.

The understanding of the basic mechanisms which govern the leucocyte changes occurring after brief stress situations makes it possible to view critically the discrepancies observed in reports by previous workers. Claims and counterclaims were not primarily concerned with the occurrence of leucocytosis following a stress situation, as the observations were fairly uniform in this respect, but rather with the differential type of cells that were accountable for the leucocytosis. The studies on epilepsy were especially at variance.[35, 47]

A review of the reports which led to the incongruity of claims brings forth evidence that the individual authors failed to direct sufficient attention to three factors, which may now be viewed as most essential for obtaining uniform results. These factors are accurate and standard time intervals in relation to the onset of stress, the occurrence of another stress situation preceding the stress under observation, and possibly the constitutional individuality of the subjects under observation.

Investigators who collected their samples within a half hour after a single stress experience were apt to find a decided lymphocytosis. In contrast, investigators who secured samples approximately one hour or later after a stress found a neutrophilic leucocytosis, as did also those who analysed samples from subjects with recurring stress (cf. Fig. 1).

The fundamental kinship of the leucocytic responses in various forms of brief stress is also carried over to the phenomena of repeatedly applied stress. The lymphopenic-neutrophilic leucocytosis after epileptic convulsions recurring at intervals of several hours is evidently only a protracted second phase of the leucocytosis of brief stress stimuli repeated sufficiently often to maintain a continuous state of adreno-cortical activity similar to that observed under conditions of protracted stress such as exposure to cold, exhaustive muscular effort, infectious illnesses, etc. The elucidation of the mechanisms underlying the protracted lymphocytosis, which occurs in some individuals in whom stress recurs at daily intervals, is not clearly evident from the few studies represented. Nevertheless, the sustained lymphocytosis to repeated stress is sufficiently universal to have occurred in four of the five types of stress reviewed, e.g. epinephrine injection, epilepsy, electric convulsive therapy, and emotional tension preceding a series of college examinations.

The extinction of the lymphocytic response after repeated injections of epinephrine, after convulsions repeated at two-day intervals, or during athletic training is another leucocytic phenomena which is not easily interpretable and will require more investigation, particularly as to its specificity for the particular stress situation. Is it for instance impossible to elicit an epinephrine response in a subject under athletic training or during electric convulsive therapy?

Review of leucocytic phenomena after brief stress situations points to the importance of time factors of the observations. To obtain meaningful results, it is necessary accurately to appraise the stress situation as being an independent, single stress, or one of a sequence of stresses; furthermore, it is necessary to time accurately the onset of the stress and the intervals of

observation. With due regard given to the time factors and singularity of the stress situation, valuable conclusions on the metabolic state of the organism may be drawn from observations of the changes in blood leucocyte levels. If the stress situation is repeated at short intervals measured in hours, the leucocyte response assumes the character of an enduring chronic stress situation, with its characteristic neutrophilic leucocytosis and extended adreno-cortical function. Spacing at intervals of one or two days permits recovery from the neutrophilic leucocytosis, in some instances leading even to a prevalent lymphocytosis; nevertheless, the influence of the previous stress is still sufficiently ingrained to preclude the occurrence of a full lymphocytic response. Spacing of identical stress situations at intervals greater than four days probably isolates the stresses sufficiently to make them independent and individual in respect to a mutual influence upon each other.

Conclusions and summary

The wealth of documentary reports which deal with the leucocyte changes during brief stress situations points to the conclusion that a similar physiological process follows each brief stress situation, whether it be as violent as the experience of an epileptic convulsion, or fear of injury, or perhaps as gentle as the mere contemplation of a tasty meal; the leucocytic response retains a similar pattern whether it expresses the consequences of already expanded effort or a mere preparedness for an internal or external adaptation.

The response of circulating leucocytes to brief stress is consistent with known mechanisms of adrenal function and their relation to stress. On the basis of pertinent observations of various authors a hypothesis is formulated that the initial lymphocytosis which follows the brief stress situation is a consequence of an epinephrine-like action—a usual concomitant of brief stress. The subsequent lymphopenic and neutrophilic leucocytosis is in turn an expression of activity of the adrenal cortex.

A protracted aftermath of the brief stress situation manifests itself in the leucocyte picture by a diminished lymphocytic response on repetition of a similar stress within two or three days, as for instance in electrically induced convulsions. In occasional subjects lymphocytosis may prevail during a series of suitably spaced brief stress situations.

Since both a lymphopenia and a neutrophilia result from adreno-cortical activity, appropriate combination of numerical values of both the lymphocyte count and the neutrophil count in a single value enhances the sensitivity

of the leucocyte count as an indicator of adreno-cortical activity. A useful expression of adreno-cortical activity is the commonly used relative lymphocyte count or a ratio of lymphocytes to neutrophils—$\frac{\text{Lymphocytes}}{\text{Neutrophils}}$.

The three most important determinants of the differential type of leucocyte count which will be found after a brief stress situation are: the duration of the stress and the time of the interval following stress; the absence or presence of a preceding stress within approximately four days; and the constitutional individuality of the subject.

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NEWS AND COMMENT

Report of the Dean

Dr. C. N. H. Long, Dean of the School of Medicine, has very kindly given permission for the reprinting of the following portions of his 1948-49 Annual Report to the President and Fellows of Yale University.

* * * *

The past year, as well as those immediately preceding it, has found the School of Medicine, along with its sister schools in this country and Canada, confronted by one of the most difficult periods in its history. These difficulties and perplexities do not rest solely on the adverse financial situation of the majority of medical schools, but in equal measure are contributed to by the uncertainties surrounding the whole question of the future of the medical care of our people. In this structure medical education is a keystone and the bitter battles at present being waged around the various health programs offered by opposing sides carry as deep implications for our future as they do for the individual who in sickness will be the responsibility of the physicians we train. . . .

. . . it is now generally agreed that a medical school can no longer confine itself to the classical forms of medical education under which the school endeavored to train good physicians and, having done so, considered its responsibilities to have been met . . . I also believe that in the future all schools will be judged by the degree to which they succeed in articulating their policies and programs with the growing social consciousness that adequate medical care for our people, regardless of economic circumstance, must in some way be provided. Such medical care in the last analysis is only as good as the physicians that the medical schools provide and cannot be furnished merely by promulgating vast compulsory or voluntary health insurance plans. The provision of adequate numbers of well-trained doctors, nurses, and technicians is the first essential in any program that seeks to improve and safeguard the health of the people.

If it be granted that new forces are in operation which will greatly increase the responsibilities of this medical school, it becomes necessary to examine our present position in this new light. These new responsibilities are being presented to us at a time when the cost of carrying out our present program is such as to have raised questions as to how long even this University could afford to undertake them. The determination of the University to make every effort to maintain the School, as expressed by President Seymour last Alumni Day, has not only heartened the faculty and administration of the School, but has enabled us to embark upon certain new programs which are in keeping with the new concept that the School should serve as the medical center of Connecticut, as well as an

educational institution dedicated to the training of physicians and the advancement of medical science. . . .

. . . knotty problems relating to ways and means of meeting the costs of present-day medical education must be solved before this School can contemplate any expansion of its programs that would involve increased costs to the University. It may be pointed out, however, that two surveys of medical education and its needs, one by the U. S. Public Health Service and one by the Association of American Medical Colleges, are in progress.

The Congress also has before it a bill that for a three-year period would make a grant of $500.00 a year for each undergraduate medical student enrolled and $1,000.00 for each student in public health. This is regarded by the sponsors of the bill as no more than a stop-gap measure pending the results of the surveys that are to be conducted on the needs of the schools. The acceptance of such assistance by this School is a question that will need careful study. However, under proper conditions there seems no reason why this type of Government grant should be more dangerous to the School than the acceptance of $500,000.00 from Government agencies for the conduct of research.

Nevertheless, the School must sooner or later receive additional support, both to maintain and increase its staff and to provide additional laboratories and the modernization of others. If the funds that are required for these purposes, as well as those necessary to reduce the large burden on general University funds, cannot be obtained from private sources, there would appear to be no alternative to Government assistance except a catastrophic reduction in our educational standards.

It is, therefore, with real interest and appreciation that some of us have met with the Committee on Medical Affairs of the University Council to place before this Committee not only the present needs of the School, but also our plans and ambitions for the future.

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[*Medical education.*] . . . It may be said that the present defects in our system of medical education are (a) the length of time required; (b) the high cost to the individual and the medical school; (c) admission requirements that are not clearly understood by the colleges furnishing premedical training; (d) variable standards of preparation of those leaving the college to enter medical school; (e) the division of responsibility for medical education between college, medical school, and hospital; (f) the inflexibility of the medical curriculum within the schools, particularly in the teaching of the first two years, and a lack of coordination and integration which prevents an intelligent anticipation of the needs of physicians in the years ahead.

It is not difficult to point out the present defects in our system of medical education but it is not an easy task to suggest changes that can be introduced to improve upon the present plan. Furthermore, piece work alterations in the present curriculum are unlikely to accomplish the major objectives that should underlie the education of physicians. Broadly speaking

these objectives are (a) to select individuals with the ability and personal characteristics required for a career in medicine; (b) to design an educational program that will impart not only a broad cultural background but also acquaint them with the basic sciences underlying the practice of medicine. This period which includes the present two preclinical years in medical school is to be regarded as the preparation for entry into the clinical period of study; (c) the clinical period, which should cover not only the last two years of the present curriculum but also whatever period is necessary to fit the individual for the general practice of medicine; (d) the final period is one in which individuals of a high degree of competence are selected and given the further training that is necessary to qualify them for specialization in some particular field of medicine.

It has already been pointed out that at present the schools have real control only over the four years of undergraduate education. Before and after this period the responsibility for the educational program rests in other hands. Nevertheless, it is the good fortune of this School that as part of a great University it has the opportunity to develop new educational programs and to experiment with these in a way denied to many of the other schools in the country.

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[*Teaching.*] The best designed medical curriculum is only as effective as the teachers who are responsible for its presentation to the students. This School of Medicine has the reputation of being primarily a research school and one where excellence in teaching is considered secondary to research productivity. Like all generalizations this is only partly true but there is enough truth in the statement to give cause for concern. . . . There are certain principles that should be continually borne in mind: (a) The primary purpose of the School is the education of physicians for general and specialized practice. For this purpose we should seek out and encourage as faculty members those individuals who are not only competent in their field but who have a gift for teaching it to others. The ability to inspire students is not an unworthy goal; indeed, great teachers are as uncommon as great investigators. It should be made clear, particularly to the younger members of the staff, that success as a teacher is a primary consideration both for appointment and promotion. (b) I have frequently been told that promotion in this School is largely based on productivity in research. If this is correct it is a deplorable policy for two reasons. In the first place it encourages the young teacher to forward his research at the expense of his students. Secondly, it assumes that competence in research is always accompanied by ability to teach. Nothing could be further from the truth. Conversely it leads to the conclusion that an individual who devotes time and care to meeting his teaching responsibilities is either indifferent to research or ignoring opportunities for its pursuit. Nor is this always true. An individual indifferent to the advancement of his subject is hardly likely to be a stimulating teacher. . . . The assurance that the School does regard eminence as a teacher as proper

reason for promotion . . . would do much to correct many of the present imperfections in the School.

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The extramural graduate program. Last year it was reported that the School would offer a series of postgraduate courses for the practicing physicians of the State. This endeavor has the support of the Connecticut State Medical Society which contributed $10,000.00 to be used for this purpose over a two-year period.

Dr. Willard, who has had the responsibility for the administration of this program, reports that the first year was far more successful than had been anticipated. . . . The experience of the past year has indicated that the program should not be carried entirely by the faculty of the School. The necessity for continued instruction of physicians, whether to prepare them for specialty board examinations or simply to keep them abreast of the times, is one that should be shared with the larger hospitals of the State and the State Medical Society. The Hartford Hospital recently inaugurated a postgraduate program under the direction of Dr. J. C. Leonard. This also had most satisfactory attendance and indicates what can be contributed to such a program by a large hospital.

Since it is the wish of the School to be a participant in a statewide program rather than to conduct one independently, arrangements have been made and approved by the Board of the School that will unite the efforts of the Hartford Hospital and the School in next year's program. It is hoped that not only will this achieve an integration in postgraduate teaching throughout the State, but will also extend the teaching resources of both institutions in this area. To ensure the closest cooperation Dr. Leonard, the Director of Medical Education of the Hartford Hospital, has been given an appointment as Associate Clinical Professor in the School and has been asked to serve as a member of the Committee on Postdoctorate Education. It is our intention to invite other qualified physicians in the State to serve as teachers in this program. By this means the program need never become burdensome for our own clinical departments, particularly if we use all the qualified men throughout the State who are willing to participate.

This whole program is another example of the expanding participation of the School in the medical affairs of the State. . . .

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Relations of the School with the Grace-New Haven Community Hospital. The New Haven Unit of the Grace-New Haven Community Hospital furnishes the clinical facilities for the instruction of the students of the School. It cannot be emphasized too often that the Hospital and the University are partners in an enterprise that is beneficial to both. For our part we have access to facilities without which the School could not have developed to its present degree. The Hospital and the community benefit from the services of the highly skilled members of the clinical departments

of the School. It is therefore a matter of great importance that there should exist not only the most cordial relationships between the two institutions, but also a clear definition of the responsibility of each partner in this coordinated program. Through the efforts of Dr. Darling, Dr. Snoke, and the members of the Board of Directors of the Hospital we have made substantial progress during the past year in the clarification of these individual responsibilities.

As is well known, there has existed for a number of years a "no man's land" in our relationship. This was largely a consequence of the evolutionary process that began in 1913 with the first agreement between the Hospital and University for the use of the clinical facilities by the Medical School. The rapid expansion of the physical facilities of the Hospital and the emergence of the School to a leading position among its kind left still further areas of financial responsibility in an uncertain state. So long as the sums involved were not great no difficulties arose, but in recent years the increasing costs of operation of both institutions finally brought them to the point where a free discussion and clarification of the whole question of individual responsibility for certain costs was essential.

Since our fortunes are so inextricably interwoven there never was any question of a preëmptory challenge from either institution to the other. It is a pleasure to report that the negotiations carried on over the last three years by Dr. Darling with Dr. Snoke and the Board of Directors of the Hospital have been marked by cordiality, a spirit of compromise, and an understanding of each other's objectives. The University has recognized that the Hospital could not be suddenly asked to assume costs which for many years had been met from the budget of the School. Any such change had to be made over a reasonable period during which the Hospital would have an opportunity to find resources to meet the costs formerly borne by the University. In keeping with this policy, the direct grant from the University to the Hospital has been reduced . . . over the three-year period. At the same time a substantial reduction in the indirect costs, largely for service laboratories, has been put into effect. This represented the salaries of technicians and supplies for service laboratories which heretofore had been charged against the budgets of the clinical departments.

Last year the costs of the laboratories of clinical microscopy, bacteriology, serology, the blood bank, electroencephalography, and electrocardiology were assumed by the Hospital. This coming year the costs of the laboratory of clinical chemistry and certain costs of the laboratory of surgical pathology will be met by the Hospital. Except for some minor items this will complete this part of the program.

The assumption of the costs of these laboratories by the Hospital does not relieve the School of the responsibility for their professional supervision. The School has designated a member of the department concerned to serve in this supervisory capacity. The various individuals so nominated have been constituted as a Laboratory Council, of which Dr. L. L. Waters of the Department of Pathology has served as chairman. Dr. Waters has

also been in charge of the laboratories of the Grace Unit since Dr. Bartlett's retirement last year. The Laboratory Council advises the Hospital authorities concerning fees, recommends to the Medical Board of the Hospital the various types of laboratory procedures that should be performed, and in general acts as a liaison group between the School and Hospital.

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Radiology. Dr. Hugh M. Wilson, after 18 years of unselfish and distinguished service to the School and Hospital, has decided to accept the directorship of the Mallinckrodt Institute of Radiology at Washington University, St. Louis. His decision to leave us is only partly tempered by the knowledge that his eminence in his field has been recognized by his appointment to what is perhaps the foremost post of its kind in the country. Dr. A. H. Janzen will become Radiologist-in-Chief to the Hospital and Associate Professor in the School. . . .

The resignation of Dr. Wilson—while deeply regretted—gave an opportunity for a recasting of the Hospital and School relationship in regard to this department. The high degree of excellence to which the department had been brought under Dr. Wilson's direction has increased the work to the point where almost the entire time of the members of the department has been occupied in hospital service functions. The Hospital has been fully aware of the heavy demands on the department and even prior to this year had added additional members to the staff at its expense. For the coming year the Hospital will assume the responsibility for the department. The School, however, will continue to make a contribution to the salaries of those individuals who will be directly concerned with the instruction of our students.

It is not the intent of the School to limit its interest in Radiology to the degree represented by this contribution. We foresee that as the University interest in Biophysics develops, further appointments will have to be made in Radiology. These individuals will be appointed for their contributions to the educational and investigative program in the subject, for from this time on it will be the responsibility of the Hospital to provide all the assistance needed to carry out the service functions of the department. Moreover, it is the intent of the School to recommend all professional members of the department for university rank, as by this means the best cooperation between School and Hospital can be maintained.

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Relations of the School with other Connecticut hospitals. It is often assumed that the association of the School with the Grace-New Haven Community Hospital is the only relationship that exists between ourselves and the hospitals of the State. In point of fact we have close associations with many other hospitals in Connecticut. These associations have been developed over a period of years by the various clinical departments and among them are the following involving an exchange of residents:

The Children's Center of the New Haven Orphan Asylum, Hamden—Pediatrics
Connecticut State Hospital, Middletown—Psychiatry
Fairfield State Hospital, Newtown—Psychiatry
Hartford Hospital, Hartford—Anesthesia, Orthopedics, and Neurosurgery
Hospital of St. Raphael, New Haven—Pediatrics
Laurel Heights (State Tuberculosis Sanatorium), Shelton—Medicine
Southbury Training School (State), Southbury—Pediatrics
Hospital for Chronic Illness (State), Rocky Hill—Medicine
Uncas-on-Thames (State Tuberculosis Sanatorium), Norwich—Medicine
Windham Community Memorial Hospital, Willimantic—Surgery

The School has agreed to new or extended affiliations with Uncas-on-Thames, Southbury, Rocky Hill, and Fairfield State Hospital for the coming year.

In addition to these affiliations the School is providing, by a recent contractual arrangement, supervision and educational services in Pathology for the Fairfield State Hospital. This is in addition to the long standing affiliations with the New Britain, Meriden, and Norwalk Hospitals.

Our relationship with the Veterans Administration Hospital at Newington has continued to be most satisfactory. In addition to the nomination of the residency, consulting, and attending staffs by the Dean's Committee, the School has had affiliated residencies in Surgery, Radiology, and Psychiatry with this hospital.

A major problem still faces many Connecticut hospitals. This lies in their difficulties in obtaining sufficient internes and residents for their services. These difficulties arise from the fact that many of these hospitals are of small size and consequently are unable to offer to their residents the kind of educational experience that is now required by the specialty boards. As a result these small hospitals lack applicants for their internships and residencies while the larger hospitals, particularly those associated with medical schools, have more applicants than they can accommodate. We receive frequent appeals for help from these hospitals in obtaining internes or to provide educational opportunities in the School for their residents. Under the present situation there is little we can do in providing internes and residents, although as pointed out above we have established affiliated residencies with a number of hospitals.

The real need is for the development of a program of cooperating central and regional hospitals throughout the State. In the larger or central hospitals are to be found many of the facilities that the smaller lack. A single central hospital could affiliate with the adjacent smaller ones and arrange a system for the interchange of internes and residents. This requires that the central hospital assume the responsibility for the operation of an educational program that will be satisfactory to the specialty boards. In addition the central hospital must be assured that the affiliated hospitals are willing to do all they can to meet these needs. This may often involve the approval of their programs by the educational director of the central hospital.

The part to be played by this School is twofold. First, as the only medical school in the State, it possesses resources in its staff and laboratories that are not found elsewhere. It should therefore be prepared to open these unique facilities to the residents of other hospitals in the State. This has already been done in part. The postgraduate program makes available courses in Anatomy, Endocrinology, and special clinical subjects that can only be obtained in the School. Second, through our teaching hospital, the Grace-New Haven Community Hospital, the School can work out a program of affiliation with the smaller hospitals in our neighborhood. A start has also been made in this area. Agreement has been reached with the Middlesex Hospital at Middletown for a cooperative program of interne training to begin July 1, 1950. Under this agreement the School will nominate and appoint to the faculty an educational director for the Hospital. His salary will be met by the Hospital and he will be expected to devote half his time to their program. Our responsibility will be in the design of a program which will offer to the internes opportunities in the School of Medicine, in addition to the program developed by the educational director in the Middlesex Hospital. We shall also be expected to use our influence to attract individuals to this program.

It is recognized that this is an experiment, the degree of success of which is not predictable at the present time. If it is successful it may serve as an example for the organization of Connecticut hospitals into cooperating and interdependent groups. Such an arrangement would to a large degree relieve them of many of their present difficulties in obtaining and training internes and residents.

In summary, our present and contemplated services to Connecticut hospitals may be enumerated as follows:

1. Assignment of internes and residents to a number of hospitals for part of their training and to assist these hospitals.
2. Providing basic science courses or other work for qualified residents of Connecticut hospitals.
3. Development of a cooperative program of interne training (at present only the Middlesex Hospital has accepted such a program).
4. Offering special postgraduate courses in community hospitals to the hospital staff.

Other relationships with the medical profession of Connecticut. The Connecticut State Medical Society has for many years had as one of its committees that known as the Committee on Coöperation with the Yale University School of Medicine. During the past four years this committee has been under the chairmanship of Dr. Thomas P. Murdock of Meriden. The representatives of the School have met regularly with this Committee which, under Dr. Murdock's guidance, has developed into a very effective liaison group between the School and the representatives of the medical profession of the State.

It is as a result of the initiative of this committee that the State Medical Society made its contribution to the program of postgraduate education.

Through other discussions in the committee the School was requested to establish a diagnostic clinic to which physicians could refer their patients. This clinic began this service last year under the direction of Dr. Hugh L. Dwyer, Jr. Without undue publicity it has already grown to an extent which is indicative of its usefulness.

I believe it is important that the School should have an opportunity to meet frequently with a committee which represents the physicians of the State. As we enter into such activities as an extended postgraduate program affiliation with other hospitals and other endeavors that have in the past not been closely associated with the program of the School, the counsel and assistance of this committee will do much to prevent any misunderstanding of its motives. It is now the policy of the School to participate, as far as is compatible with our primary educational responsibilities, in the health and welfare programs of the State. The active interest and support of every physician in the State would be of immeasurable advantage to us. In the past the School has not participated in the medical affairs of the State to the degree that is compatible with its position as the only medical school in Connecticut for while our objectives and responsibilities are not limited to this State, to ignore the many opportunities to advance our own position that are to be gained by such association would hardly seem the path of wisdom.

I should like to emphasize again the debt we owe to those members of the profession in the State who serve the School through their appointments as clinical or visiting members of the staff. These physicians serve without compensation, but contribute incalculably to our educational program by their services in the wards and dispensaries of the hospital. The full-time system of medicine is the nucleus upon which all university clinical departments should be based, but no university department could or should endeavor to base its whole instructional program upon full-time members of the faculty. In the first place, complete coverage in all departments would be inordinately expensive. Secondly, it is undesirable since the physician in private practice brings to the student additional points of view which serve to broaden his educational experience.

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Research in the School of Medicine. Last year the School received over one million dollars in support of the research programs carried on in the various departments. Of this sum, nearly half was from agencies of the Federal or State Government, while the remainder was donated by various private philanthropic agencies and individuals. To these sums must also be added $109,955.54 which was disbursed from the various research and fellowship funds of the School.

This lavish support of the research programs of the staff is to be contrasted with a deficit of $628,904.70 during 1947-48 in the operation of the School and, indeed, the total exceeds by over $200,000.00 the sum available in the budget for the support of the educational programs of the

departments. In the case of some departments the research funds available were two or three times larger than those derived from the University. The inflow of research funds of this magnitude has brought to the School cause for both satisfaction and concern.

In the first category is to be placed the recognition that this School is one of the foremost medical research centers in the country, particularly in such fields as cancer and poliomyelitis. The possession of these funds has not only enabled the faculty to expand and accelerate their research programs but has also meant the temporary addition to the departments of many able young men and women whose presence here was made possible by these grants. The beneficial results of the intensive research carried on in this School and others by means of these funds will ultimately be reflected in an increased ability to deal with human disease and by significant additions to our understanding of biological processes in general.

On the other side of the ledger the disproportion between funds available for the research and educational programs of the School has led to the opinion—both within and without the School—that our major purpose was the creation of a research institute rather than a center of medical education. This, I am sure, is not a conscious intent on the part of the faculty but rather a result of the contrast between the swollen budget of the research activities of the departments and the impoverishment of their University resources. . . . It is evident that ways must be found to redress the balance between teaching and research in the School. . . . This School, and probably many others, is now unable to accommodate more research programs than it is doing at present while, at the same time, it is faced with a decline in its high educational standards unless funds are found adequately to reimburse its teachers and to add to their numbers.

BOOK REVIEWS

MANUEL TECHNIQUE DE MICROBIOLOGIE ET DE SEROLOGIE. By A. Calmette, A. Boquet, L. Negre, and J. Bretey. Masson et Cie., Paris, 1948. 727 pp. 1900 fr.

This fourth, thoroughly revised, edition of Calmette's well-known manual aims to bring together the maximum of information which will be of value to the bacteriologist in his daily work. It is based largely on the techniques utilized at the Pasteur Institute in Paris. The topics included differ from those found in similar manuals in this country which are usually texts of laboratory diagnosis and include fields deliberately omitted in the present manual (e.g., urinalysis). The book is essentially a companion volume to a text on bacteriology. Within its stated limits, coverage is broad, and the book contains an extraordinary diversity of useful data and procedures. An unfamiliar apparatus noted is the centrifuge tube, designed by Bretey, which houses a cover slip at the tip. Scanty sediments intended for morphological study are said to be harvested with less loss than by usual methods.

The American reader will be struck by the amount of media and apparatus which he purchases from supply houses, whereas his French colleague prepares the same material in his own laboratory. This is probably true, although perhaps to a lesser extent, in other countries. It follows that, in our scientific papers, much of our "Materials and Methods" section must be largely incomprehensible outside of our national boundaries. "Nutrient agar *Difco* 1.5 per cent, pH 7.4, 4 grams in one liter of distilled water" for example, has provided not only insufficiently detailed, but actually misleading information.

Future editions would be more useful were the bibliography enlarged so that for each topic additional information could easily be obtained.

DAVID WEINMAN

EVALUATION OF CHEMOTHERAPEUTIC AGENTS. Edited by Colin M. MacLeod. Columbia University Press, New York, 1949. 205 pp. $4.00.

This is the second in the series of volumes to come from symposia held by the recently established Section on Microbiology of the New York Academy of Medicine. As stated by Gregory Schwartzman in the foreword, "the programs of meetings of the Section are arranged to deal with single topics in the various aspects. . . . The participants present extensive reviews in their particular field of endeavor, thus bringing correlated and authoritative scientific data and concepts to the attention of the microbiologists and allied laboratory and clinical investigators." In this volume an evaluation of chemotherapeutic agents is undertaken from many, rather general and fundamental aspects. The topics included can be summarized as follows: The relation of chemotherapeutic activity to drug concentrations in blood

and to renal clearance; the binding of agents to proteins; microbial resistance; defense mechanisms of the host and the nature of the lesions in relation to chemotherapy; chemoprophylaxis of meningococcal, bacillary dysentery and streptococcal infections; chemotherapy in malaria and in rickettsial and viral diseases; transcutaneous anti-biotic treatment; and experimental and clinical evaluation of chemotherapeutic agents in cancer. The contributors to the fourteen different sections are experts in their particular field and hence this volume should be of great value to anyone, laboratory worker or clinician, interested in the fundamental aspects of chemotherapy. Each section is followed by an adequate bibliography.

W. W. ENGSTROM

HUMAN EMBRYOLOGY AND MORPHOLOGY. By Sir Arthur Keith. Williams & Wilkins, Baltimore, 1948. ix + 690 pp. $10.00.

This being the sixth edition, extensively revised, of a book which first appeared in 1901, comments on its continuing usefulness are superfluous. It is enough to say that the reader will find a detailed account of most aspects of human embryology and that developmental and adult aspects of anatomy are constantly compared. The style is perhaps more labored than in comparable texts by American authors. A few of the numerous illustrations suffer from excessive reduction. Indexing, printing, and binding have been well done. Numerous notes and references increase the book's utility. This reviewer was disappointed that, although there are three chapters on the urogenital system, he found little on the development of the testis and almost nothing on the development of the ovary.

THOMAS R. FORBES

ACCLIMATIZATION IN THE ANDES. By Carlos Monge. Translated by Donald F. Brown, with an introduction by Isaiah Bowman. The Johns Hopkins Press, Baltimore, 1948. xix + 130 pp. $2.75.

Despite the intense interest during the past five decades in the physiological effects of the low pressures at high altitudes enforced either by the ascent of mountains or in aircraft, scant attention has been given to the equally important mechanisms of acclimatization that follow the movement of a population from low to high altitudes. Dr. Monge, Director of the Institute of Andean Biology, is one of the few to focus his attention on these mechanisms of acclimatization through a study of Andean man as a climato-physiological variant of the human race. Some of the results of the study are presented in this monograph in a form attractive to layman and scientist, be he interested in physiology, sociology, or anthropology. Here is outlined the evidence that acclimatization can and has taken place in a human population through the acquisition of the ability to reproduce at altitudes in which their ancestors were infertile. Here, too, is the record of the customs that provide the social basis for the natural selection which has resulted in an acclimatized people. This evidence, drawn from the

historical records of four hundred years, is worthy of careful consideration by a wide audience, for it brings hope for the future of mankind through continued acclimatization.

DONALD H. BARRON

PERSPECTIVES IN MEDICINE. The March of Medicine, 1948. Columbia University Press, New York, 1949. 163 pp. $2.50.

This book is a collection of the thirteenth series of the Laity Lectures of the New York Academy of Medicine. Delivered for the benefit of those interested in the medical and teaching professions and also in the related fields of social service and public health, the essays have a common theme which emphasizes the interrelationship of medical research with the public and its elected representatives in the government.

The lectures deal mainly with contemplated research, such as the potentialities of radioactive isotopes in medical investigations which are discussed by Lewis L. Strauss of the Atomic Energy Commission. However, they include such diverse topics as "Food and Civilization," psychiatric problems in ageing, and cancer research.

This little book contributes to the background knowledge of workers in the biological and physical sciences. The discussion of the "Inter-Relation of Pure and Applied Science in the Field of Medicine" by James B. Conant, President of Harvard, is of fundamental importance to all research workers. It presents to the laity with force and clarity the justification for the public and private financial support of all fields of medical research, extending from the basic sciences through the applied research laboratories to the clinical research workers in the hospitals and public health clinics. It is recommended to anyone who is concerned with scientific progress.

ROBERT E. COOKE

INTROGRESSIVE HYBRIDIZATION. By Edgar Anderson. John Wiley and Sons, New York, 1949. ix + 109 pp. $3.00.

Recent years have seen a resurgence of evolutionary studies: the establishment of a special journal and the publication of Julian Huxley's *Evolution, the Modern Synthesis* are noteworthy events. And now comes Anderson's little book, casting a searchlight beam on a neglected, possibly fundamental process in evolutionary adaptation—exchange of genes between species, races, etc. Examples of great cogency are presented in iris, sunflower, lettuce, and other familiar plants. Anderson points out that one of the prime agencies in encouraging the start of evolutionary developments from hybridization is great disturbance of the environment. Of course the hybrids must have at least a small amount of fertility for further diversification among descendants.

This book is not only an exposition of new information but a fervent call for more research along this line. But he says that for showing relations between the main variables in populations "the methods of conventional

biometry are laborious and inefficient." In their place he describes methods, admittedly crude, which serve well for exploratory investigations. These include standardized photographs, pictorialized scatter diagrams, ideographs, hybrid indices, and "radiate indicators." Properly used, these seem not to be laborious or inefficient; more important perhaps is their appeal to many non-mathematical biologists. Anderson shows interesting examples of their use in deducing the characteristics of an unknown species which had introgressively hybridized with another.

The most disappointing feature of the book is its strict limitation to plants; probably the author was wise in recognizing his limitations, but the possibilities in zoology and anthropology are most intriguing. On the other hand, Anderson's enthusiasm and preoccupation with multiple factor inheritance may have somewhat distorted his perspective. No doubt Burbank would have chortled. At any rate, here seems to be a handbook for the new hybridist.

W. F. HOLLANDER

ISOTOPIC CARBON. By Melvin Calvin, Charles Heidelberger, James C. Reid, Bert M. Tolbert, and Peter E. Yankwich. John Wiley & Sons, Inc., New York, 1949. xiii + 376 pp. $5.50.

It is remarkable, and probably significant, that a special text dealing with the use of carbon isotopes in biochemistry has appeared in the brief time which has elapsed since the carbon isotopes have become generally available. The haste with which this book was written is apparent in the lack of polish of the style of its writing. In spite of this shortcoming, the important subjects of measurement of mass and radioactivity, organic synthesis, sample preparation, and criteria of purity have been carefully, although briefly, treated.

There is little doubt in the reviewer's mind that many biochemists are inhibited, in their desire to employ tracer techniques, by the problems of organic synthesis with which they must deal. From this point of view *Isotopic Carbon* is encouraging and, indeed, stimulating. Often overlooked, but of equal importance, are those aspects of tracer methodology which are concerned with the establishment of purity of materials. This subject is adequately outlined in a separate chapter of the text.

In view of the manipulative skill required in order to synthesize organic isotopic compounds, and the painstaking techniques necessary to establish the purity of labelled material, the reviewer does not share the opinion of the authors, who are "convinced that isotopic carbon as a tracer will soon find its place as a routine tool . . ." It is already apparent that a new species of chemist has arisen to deal exclusively with the unique problems which are continually appearing in tracer work. Nevertheless, *Isotopic Carbon* will encourage many who are serious in their desire to investigate their particular problems by means of isotope methods.

H. D. HOBERMAN

THE USES OF PENICILLIN AND STREPTOMYCIN. By Chester Scott Keefer. University of Kansas Press, Lawrence, Kansas, 1949. 72 pp. $2.00.

This small volume, which consists of the three Porter Lectures, is an excellent summary of the knowledge of clinical applications of penicillin and streptomycin. The first section is devoted to penicillin—mechanism of action, forms available, dosage, reactions, and results of therapeutic as well as prophylactic use in a variety of infectious diseases. The second section discusses, in concise form, the theoretic and practical aspects of streptomycin therapy. The third section presents a brief and interesting account of the story of anti-bacterial agents derived from microbes.

ROBERT H. GREEN

PLANT PHYSIOLOGY. By Meirion Thomas. J. and A. Churchill, London, 1947. xi + 504 pp.

This third edition of an English textbook, published in 1947, has been expanded particularly on the biochemical side. An extensive section on enzymes, particularly oxidative enzymes, zymase, and phosphorylases, and new material on photosynthesis and the metabolism of di- and tricarboxylic acids are important additions. The first part, Protoplasm, deals with visible structures, physico-chemical and biochemical properties of living material, with a proper, strong emphasis on plants. Part two, The Absorption, Translocation, and Elimination of Water, Solutes, and Gases, deals generally with the movements of materials, and includes osmotic phenomena, soils, and the usual problems of water and solute. Part three, Nutrition and Metabolism, includes a discussion of general problems and methods, as well as specific chapters on photosynthesis, respiration, and the polycarboxylic acids. Part four, Growth and Movement, includes, besides these subjects, a chapter on growth substances. Two appendices offer basic information about organic compounds and physical chemistry, and a third one includes a bibliography of 344 references and author and subject indices. The volume gives a good picture of the general physiology and biochemistry of plants at an intermediate advanced level. It should serve as a satisfactory textbook in plant physiology and as a useful reference volume for workers in other biological fields.

R. W. GERARD

EXPOSÉS ANNUELS DE BIOCHIMIE MÉDICALE. Edited by M. Polonovski. Masson et Cie., Paris, Series 8 and 9, 1948 and 1949. 368 and 319 pp. 1000 fr.

These volumes constitute an excellent series of review articles which have not up to this time been widely read by English or American scientists. The earlier issues of the "Exposés" were produced mainly during the war and suffered heavily from the writers' isolation from the world literature. In the latest two issues there are few signs of parochialism left. The articles

are written by the most eminent French-speaking biochemists. Critical and comprehensive reviews have been written on the following subjects:

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